PREDICTORS AND THE EFFECTS ON MATERNAL AND BIRTH OUTCOMES OF *PLASMODIUM FALCIPARUM* INFECTION IN THE FIRST TRIMESTER AMONG NULLIPAROUS WOMEN FROM THE DEMOCRATIC REPUBLIC OF THE CONGO, KENYA, AND ZAMBIA

Sequoia Iris Leuba

A dissertation submitted to the faculty at the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Epidemiology in the Gillings School of Global Public Health.

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Approved by:

Daniel Westreich

Kimberly A. Powers

Steven M. Taylor

Andy Olshan

Melissa Bauserman

ABSTRACT

Sequoia Iris Leuba: Predictors and the effects on maternal and birth outcomes of *Plasmodium falciparum* infection among nulliparous women from the Democratic Republic of the Congo, Kenya, and Zambia
(Under the direction of Daniel Westreich)

Protective measures against malaria during pregnancy are rarely initiated until the second trimester, leaving at-risk pregnancies unprotected from malaria infection in the critical first trimester.

This dissertation details the first large-scale multi-site study of malaria in the first trimester, and is nested within the NICHD Global Network's trial of low-dose aspirin for the prevention of preterm delivery in nulliparous women with a singleton pregnancy (the ASPIRIN Trial). This dissertation aimed to (1) characterize the factors which increase the likelihood of malaria in the first trimester and (2) estimate the causal effects of malaria in the first trimester on adverse maternal and birth outcomes.

Aim 1 first calculated the parasite prevalence in the first trimester and found that among 485 Congolese women it was 62.9%, among 677 Kenyan women it was 37.8%, and among 351 Zambian women it was 6.3%. Kenyan women younger than 20 years old were more likely to have malaria in the first trimester (prevalence difference (PD) = 0.17 [99% confidence interval (CI): 0.07, 0.26]), and across all three study countries, lower (no secondary education) vs. higher overall educational attainment was

associated with higher prevalence of malaria in the first trimester (summary PD = 0.09 [99% CI: 0.01, 0.17])

Aim 2 found higher prevalences of preterm delivery (adjusted PD (aPD) = 0.06 [99% CI: -0.04, 0.16]) and low birth weight (aPD = 0.07 [99% CI: -0.03, 0.16]) among Congolese women with malaria in the first trimester, and higher prevalence of anemia in late pregnancy among Kenyan (aPD = 0.05 [99% CI: -0.06, 0.17]), Zambian (aPD = 0.07 [99% CI: -0.12, 0.36]), and Congolese (aPD = 0.04 [99% CI: -0.09, 0.16]) women who had malaria in the first trimester.

These findings suggest that malaria in the first trimester is very common in areas of high transmission, and is associated with higher prevalences of preterm delivery and low birth weight in an area of high transmission, and with higher prevalence of anemia in late pregnancy at all three sites. These results suggest a need to improve strategies to prevent and treat malaria infections in the first trimester.

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LIST OF ABBREVIATIONS

ANC	Antenatal care
ASPIRIN	Aspirin Supplementation for Pregnancy Indicated Risk reduction In Nulliparas
BMI	Body-mass index
CI	Confidence interval
CSA	chondroitin sulfate A
DAG	Directed acyclic graph
DBS	Dried blood spot
DCC	Data Coordinating Center
DRC	Democratic Republic of the Congo
GA	Gestational age
GN	Global Network
HRP2	Histidine-rich protein 2
INTERGROWTH	The International Fetal and Newborn Growth Consortium
IPT-SP	Intermittent preventive therapy in pregnancy with sulfadoxine-pyrimethamine
IRS	Indoor residual spraying
ITN	Insecticide-treated net
LLIN	Long-lasting insecticide-treated net
MNH	Maternal and newborn health
NICHD	*Eunice Kennedy Shriver* National Institute of Child Health and Human Development
P. falciparum	*Plasmodium falciparum*

P. vivax	*Plasmodium vivax*
P25	25th percentile
P75	75th percentile
PD	Prevalence difference
pfldh	*Plasmodium falciparum* lactate dehydrogenase
PfPR2-10	*Plasmodium falciparum* parasite rate among children aged 2-10
PR	Prevalence ratio
qPCR	quantitative polymerase chain reaction
RDT	Rapid diagnostic test
RECIPAL	REtard de Croissance Intra-uterine et PALudisme
REF	Reference
SD	Standard deviation
SES	Socioeconomic status
SP	Sulfadoxine-pyrimethamine
STOPPAM	Strategies to Prevent Pregnancy-Associated Malaria
VAR2CSA	Variant surface antigen mediating adherence to chondroitin sulfate A
WHO	World Health Organization

CHAPTER I: SPECIFIC AIMS

In malaria-endemic countries, an estimated 1 in 4 women are infected with malaria during their pregnancy, and this infection can cause maternal anemia, preterm birth, stillbirth, and low birth weight.[1] Despite these adverse effects, malaria in pregnancy is difficult to identify and thus treat because pregnant women frequently have parasite densities below the detection limit of commonly available diagnostic tools. In addition, prevention strategies including the use of intermittent preventive therapy in pregnancy with sulfadoxine-pyrimethamine (IPT-SP) and the use of insecticide-treated nets (ITNs) are challenging to initiate for women early in their pregnancy.[2] IPT-SP is not recommended in the first trimester of pregnancy because of potential teratogenic effects, and most pregnant women do not use ITNs because of discomfort associated with sleeping under the ITN, difficulties in hanging the net, and/or lack of ownership of ITNs.[3–8] These challenges to diagnosing, treating, and preventing malaria leave women under-protected from malaria in the first trimester.

Despite the difficulties in treatment and prevention of malaria, the first trimester might represent a critical time for intervention to prevent the negative consequences of malaria in pregnancy. Placentation is particularly sensitive to pathology in the first trimester, and malaria in early pregnancy could inhibit trophoblast invasion and cause disruptions in placentation.[9,10] In a study of 68 mostly multigravidae women, malaria in

early pregnancy was associated with a negative impact on placental vascular development and consequent pregnancy outcomes.[11,12]

Changes in placental vascularity would be even more pronounced among primigravidae and among women living in high malaria transmission areas.[11] Women in their first pregnancy are particularly unprotected from malaria, as women naturally acquire resistance to malaria in pregnancy in successive pregnancies through the development of antibodies that inhibit binding of *Plasmodium falciparum*-infected erythrocytes to the placenta.[12,13] Thus, primigravid and nulliparous women are most vulnerable to chronic placental infection, which is associated with low birth weight and maternal anemia.[13–17] Although malaria in the first trimester could lead to adverse effects on maternal and birth outcomes, especially among women in their first pregnancy, research in this under-protected period is limited because most studies are limited to observations in the second trimester when women begin receiving antenatal care (ANC).

The current proposal is the first large-scale multi-site study of malaria in the first trimester, and is nested within the NICHD Global Network's trial of low-dose aspirin for the prevention of preterm delivery in nulliparous women with a singleton pregnancy (the ASPIRIN Trial).[18,19] As the ASPIRIN trial specifically enrolled nulliparous women in the first trimester, this sub-study leveraged this opportunity to characterize predictors and estimate causal effects on maternal and birth outcomes of malaria in the first trimester.

At enrollment, three dried blood spots were obtained from 1,513 pregnant women in the three sub-Saharan African sites of the Global Network (Democratic Republic of the Congo (DRC), Kenya, and Zambia). We analyzed dried blood spots to determine

Plasmodium falciparum infection, and then linked these results to data collected from ASPIRIN trial participants. The overall objective of this study was to characterize predictors of malaria in the first trimester, and estimate causal effects of malaria in the first trimester on adverse maternal and birth outcomes.

AIM 1: Characterize the factors which are associated with increased likelihood of malaria in the first trimester. Limited research studying malaria in early pregnancy suggest that predictors of malaria in early and late pregnancy include maternal age, socioeconomic status, and seasonality.[9,20–23] The ASPIRIN trial collected data on potential predictors including age, height, body-mass index (BMI), education, socioeconomic status, and seasonality. We characterized the factors which are associated with increased likelihood of malaria in the first trimester. We hypothesized that lower overall educational attainment (no secondary education) was associated with increased likelihood of malaria in the first trimester.

AIM 2: Estimate the causal effect of malaria in the first trimester on adverse maternal and birth outcomes. While the effect of malaria in the second and third trimester on adverse birth outcomes has been well established, the effects of malaria in the first trimester are still unclear. The ASPIRIN trial collected data on adverse maternal outcomes including anemia in late pregnancy and hypertensive disorders in pregnancy, and on birth outcomes including preterm birth, small for gestational age, low birth weight, and perinatal mortality. We estimated the causal effect of malaria in the first trimester on adverse maternal and birth outcomes. We hypothesized that malaria in the first trimester would increase the risk of all the adverse maternal and birth outcomes assessed.

This sub-study of the ASPIRIN trial provides a unique opportunity to study nulliparous pregnant women in the DRC, Kenya, and Zambia to address the important dearth in knowledge on the predictors and effects on maternal and birth outcomes of malaria in the first trimester. Understanding predictors of malaria in the first trimester could lead to better prevention strategies. Estimating the causal effect of malaria in the first trimester on maternal and birth outcomes will inform policy efforts to shift ANC to begin in the first trimester, and drive research developments on prophylactic malarial medications for use in the first trimester.

CHAPTER II: BACKGROUND

Malaria

Malaria is a serious global health issue with an estimated 228 million cases worldwide causing 411,000 deaths in 2018.[24] Malaria is an acute febrile illness that is caused by the *Plasmodium* parasite and is transmitted by the bites of an infected *Anopheles* mosquito.[24]

There are five species of *Plasmodium* infect humans, and the two main species that contribute to malaria infection are *Plasmodium falciparum* and *Plasmodium vivax*.[25] *P. falciparum* is the predominant species in sub-Saharan Africa, while *P. falciparum* and *P. vivax* coexist in Southeast Asia, Western Pacific, and South America.[25] In 2018, *P. falciparum* caused 99.7% of estimated malaria cases in sub-Saharan Africa and is the most deadly malaria parasite globally.[24]

Malaria can lead to severe illness including anemia, acute pulmonary edema, hypoglycemia, and can lead to death.[24,26] To prevent malaria, the World Health Organization (WHO) supports the use of insecticide-treated nets/long-lasting insecticide-treated nets (ITNs/LLINs) and indoor residual spraying (IRS).[2] To prevent malaria in pregnancy, WHO recommends intermittent preventive therapy during pregnancy with the antimalarial drug sulfadoxine-pyrimethamine (IPT-SP) monthly starting in the second trimester.[2]

Africa bears a disproportionate malaria burden with 93% of global malaria cases and 94% of global malaria deaths in 2018 (an estimated 213 million cases and 380,700 deaths caused by malaria).[24] Almost all of the estimated 213 million cases of malaria in Africa are caused by *Plasmodium falciparum*.[24] Nearly 85% of malaria deaths globally in 2018 occurred in 21 countries including the Democratic Republic of the Congo, Kenya, and Zambia.[2]

Malaria in the Democratic Republic of the Congo, Kenya, and Zambia

The Democratic Republic of the Congo (DRC) accounted for 12% of the malaria cases and 11% of the malaria deaths worldwide in 2018 (about 27 million cases and 45,000 deaths).[2] In the DRC, malaria transmission occurs throughout the year.[2] In 2017, 97% (78.9 million) of the population lived in an area of high malaria transmission (>1 case per 1000 population) and 3% (2.4 million) of the population lived in an area of low malaria transmission (0-1 case per 1000 population).[27] In the provinces of Nord and Sud Ubangi, located in the Northwest of the DRC, malaria transmission is high and ranges from 10 to over 300 confirmed cases of malaria per 1000 population (**Figure** 2.1).[27]

Figure 2.1: *P. falciparum* malaria cases in the DRC, 2017
In Nord and Sud Ubangi, located in the Northwest of the DRC, malaria transmission is high and ranges from 10 to over 300 confirmed cases of malaria per 1000 population.

Figure obtained from the WHO's Country Profiles for Malaria: Democratic Republic of the Congo.[27]

Kenya accounted for 2% of the malaria cases and 3% of the malaria deaths worldwide in 2018 (about 3.6 million cases and 12,000 deaths).[2] In 2017, about 70% (34.9 million) of the population lived in an area of high malaria transmission (> 1 case per 1000 population), and about 30% (14.8 million) of the population lived in an area of low transmission (0-1 case per 1000 population).[28] Malaria transmission can be as high as 300 cases per 1000 population.[28] In western Kenya in the counties of Busia, Bungoma, and Kakamega, malaria transmission is high and ranges from 10 to over 300 confirmed cases of malaria per 1000 population (**Figure** 2.2).[28] In this Lake Victoria region, malaria prevalence is 27% and ITNs are the primary prevention tool.[29]

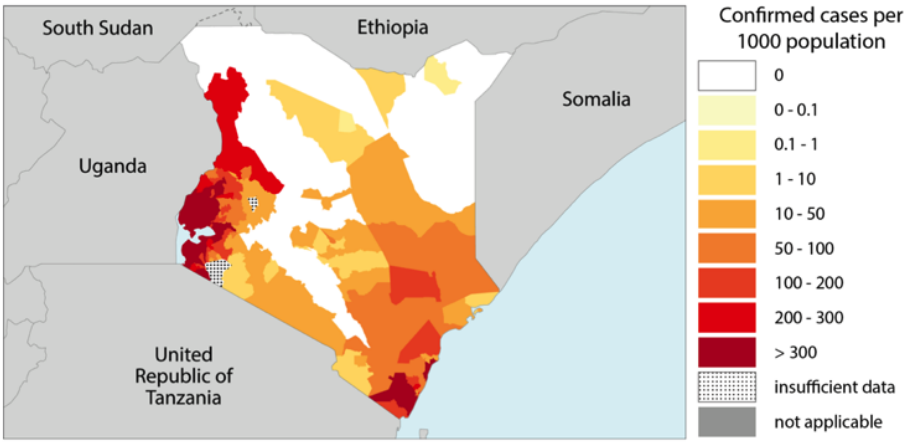


Figure 2.2: *P. falciparum* malaria cases in Kenya, 2017
In the counties of Busia, Bungoma, and Kakamega, located in western Kenya, malaria transmission is high and ranges from 10 to over 300 confirmed cases of malaria per 1000 population. Figure obtained from the WHO's Country Profiles for Malaria: Kenya.[28]

Zambia accounted for 1% of the malaria cases and 2% of the malaria deaths worldwide in 2018 (about 2.7 million cases and 7,500 deaths).[2] In Zambia, malaria transmission occurs throughout the year.[2] In 2017, all 17.1 million people lived in an

area of high malaria transmission (>1 case per 1000 population), and malaria transmission occurs throughout the year.[30] In the districts of Kafue and Chongwe, located in southern Zambia, malaria transmission ranges from 10 to 50 confirmed cases of malaria per 1000 population (**Figure** 2.3).[30]

Figure 2.3: *P. falciparum* malaria cases in Zambia, 2017
In the districts of Kafue and Chongwe located in southern Zambia, malaria transmission ranges from 10 to 50 confirmed cases of malaria per 1000 population. Figure obtained from the WHO's Country Profiles for Malaria: Zambia.[30]

A summary of the WHO recommended malaria prevention strategies (i.e., ITNs, IRS, and IPT-SP) and their implementation in DRC, Kenya, and Zambia is presented in **Table** 2.1.[2,27,28,30]

Table 2.1: Summary of malaria prevention strategies in DRC, Kenya, and Zambia

	DRC	Kenya	Zambia
Free distribution of ITNs	since 2006	since 2006	since 2005
Population with access to ITN (%)	71%	74%	79%
IRS recommended	since 2007	since 2003	since 1964
People protected by IRS (n)	111,735	1,833,860	6,436,719
IPT-SP used	since 2004	since 2001	since 2001
Pregnant women with 3+ doses IPT-SP (%)	30%+	<30%	30%+

Malaria in pregnancy

In sub-Saharan Africa, an estimated 39 million pregnancies occurred in 2018, of which 29% or 11 million pregnancies were exposed to malaria infection.[2] Malaria in pregnancy is associated with negative health outcomes including maternal anemia, low birth weight, preterm birth, and fetal loss.[13] *P. falciparum*-infected red blood cells sequester in the placenta, while *P. vivax*-infected red blood cells have not yet been documented to sequester in placenta.[25] *P. falciparum* and *P. vivax* can lead to pregnancy complications, but the consequences of infection in pregnancy with *P.* vivax is generally less severe.[25]

Malaria in pregnancy in DRC, Kenya, and Zambia

To prevent malaria in pregnancy, the World Health Organization recommends prompt diagnosis and treatment of malaria, IPT-SP, and ITNs/LLINs.[2]

Despite moderate coverage of four or more antenatal care (ANC) visits and of use of three or more doses of IPT-SP during pregnancy as recommended by WHO, the DRC has one of the highest burdens of malaria in pregnancy worldwide.[2,31–33] Each year, 3.5 million pregnancies are at risk of malaria infection, and an estimated 1 million births are affected by malaria.[2,31–33]

In Kenya, there is moderate coverage of four or more ANC visits, but use of three or more doses of IPT-SP is low.[2] ITNs/LLINs are distributed free of charge, and are distributed through antenatal care visits, well baby clinics, and through mass campaigns.[2]

In Zambia, there is moderate coverage of both four or more ANC visits and of use of three of more doses of IPT-SP during pregnancy.[2]

Definitions of malaria in pregnancy

Malaria in pregnancy is defined as a *Plasmodium* infection (primarily *P. falciparum* but includes *P. vivax,* etc.) detected at any point in pregnancy. Most research in malaria in pregnancy recruit women starting at their first ANC visit which mostly occurs in the second trimester. Thus, most malaria in pregnancy research studies infections from the second trimester onwards.

Malaria in early pregnancy in the literature is generally defined as a *Plasmodium* infection detected in the first half of pregnancy. Malaria in the first trimester is a more restrictive definition and is defined as a *Plasmodium* infection detected in the first trimester (<14 weeks of gestational age).

Parity is defined as the number of live and stillbirths, and does not include miscarriages.[34] Nulliparous women have never delivered a fetus that has reached the age of viability; thus, women are nulliparous until they deliver a stillbirth or a live birth.[34] Primiparous women have delivered their first stillbirth or live birth, and multiparous women have delivered more than one stillbirth or live birth.[34]

Gravidity is defined as the number of previous pregnancies, and includes miscarriages.[34] Primigravidae women are pregnant for the first time, and multigravidae women have been pregnant more than once.[34]

Measuring malaria in pregnancy

In non-pregnant women, malaria parasites are found in the peripheral blood circulation and sequester to endothelial cells.[35,36] In pregnant women, parasites can be found in peripheral blood and placental circulation, and sequester in the placenta along with other organs or tissue.[13,36] This sequestration in the placenta is mediated by a

parasite protein expressed on the surface of the infected red blood cell.[12] This protein adheres to chondroitin sulfate A (CSA) which is expressed on the syncytiotrophoblast (the layer of cells in the placenta lining the maternal blood spaces), and thus this parasite protein is called VAR2CSA (variant surface antigen mediating adherence to chondroitin sulfate A).[12] By expressing a different antigen variant VAR2CSA, the *P. falciparum* parasites sequester in the intervillous spaces of the placenta (known as placental malaria) and avoid splenic clearance.[13,36,37] Because *P. falciparum* parasites can accumulate in the placenta but be absent or undetectable in peripheral blood, diagnosing malaria in pregnancy is difficult.[36,37] These undetectable low parasite densities may be asymptomatic but can still have serious negative health impacts on the mother and her fetus.[3,37]

Tools used to measure malaria in pregnancy vary depending on whether it is placental or peripheral malaria. Placental malaria can be measured through placental blood microscopy and placental histology.[17,38,39] Peripheral parasitemia can be measured through blood smear light microscopy, rapid diagnostic tests (RDTs), and molecular detection techniques including polymerase chain reaction (PCR).[40,41] The sensitivity and specificity of tests are based on parasite density, with lower sensitivity at lower parasite densities.[36]

Placental parasitemia

Placental malaria is defined as malaria parasites or hemozoin pigment (a breakdown product of hemoglobin formed from digestion by blood-feeding parasites including *Plasmodium*) present in placental blood or tissue, and can be measured through placental blood microscopy or placental histology.[17,36] Placental blood

microscopy identifies through light microscopy microscopic parasite infections in placental blood collected from the intervillous spaces of the placenta.[36,42,43] However, the gold standard for placental malaria is histological observation of infected erythrocytes (red blood cells) or hemozoin pigment in the intervillous spaces of the maternal side of the placenta.[17,36] These placental histology slides are classified based on whether there are parasites in the placenta (active infection), parasites and pigment in the placenta (active chronic infection), only pigment in the placenta (past infection), or no parasites or pigment in the placenta (no infection).[36]

P. falciparum-infected erythrocytes avoid splenic clearance by sequestering in the placenta.[17] This sequestration of *P. falciparum* parasites in the placenta can occur in the absence of detectable peripheral parasitemia, making detection of malaria in pregnancy and consequent treatment difficult.[17,44] Placental histology is more sensitive than placental and peripheral blood film examination, and is the gold standard for placental malaria.[17,37] However, histology is not suitable for all situations or study designs as it (and placental blood) can only be examined after delivery and does not provide information when in pregnancy malaria infection is the most harmful to birth outcomes.[36,39,43] Thus, peripheral blood infection is used to infer placental infection before delivery, and is the only way to detect malaria infection during pregnancy.[36,37]

Peripheral parasitemia

Peripheral parasitemia in pregnancy is defined by the presence of *P. falciparum* parasites in the peripheral blood with or without placental infection.[36] In high malaria transmission areas, the parasitemia levels among pregnant women can fluctuate and be under the level of detection of common methods.[3] Therefore, the absence of detecting

malaria parasites in peripheral blood does not exclude infection or sequestration of parasites in placental tissue.[3,43] While using peripheral samples to detect malaria may not be the most accurate method, it is the only method possible during pregnancy (i.e., before delivery).[43]

The main methods for detecting peripheral blood malaria parasitemia in pregnant women are blood smear light microscopy, RDTs, and molecular detection.[40,41] These diagnostic methods vary in accuracy based on the lower limits of detection of *P. falciparum* parasites, and generally the lower limits of detection are highest for RDTs, lower for trained and well-equipped light microscopy, and lowest for PCR.[45]

Measuring peripheral parasitemia using light microscopy

Malaria can be defined using light microscopy as a peripheral blood smear positive for blood-stage asexual parasites.[37,46] Venous or capillary blood is collected to make thick and thin smears, and are stained and examined microscopically to detect the presence of malaria parasites.[36] This blood smear light microscopy conducted by an experienced technician with appropriate equipment has a detection threshold of 15 parasites per uL of blood.[3] This threshold is well below the density threshold where patients present with symptoms, and therefore is sufficient for those pregnant women presenting with symptoms.[3,5] However, most infections during pregnancy are asymptomatic with low parasite densities and therefore are often not detected by microscopy.[3,5] These submicroscopic infections (i.e., parasitemia not detected by microscopy but detected through more sensitive methods) have been associated with anemia, low birthweight, and premature births in some studies but not others.[4,5] The low

sensitivity of light microscopy prevents identification of pregnant women who may need malaria treatment to prevent further harm to the mother and the fetus.[3]

Measuring peripheral parasitemia using rapid diagnostic tests

An alternative to peripheral blood microscopy with reasonable accuracy are RDTs, a more recent method that is quick and easy to use in inaccessible areas.[13,36] While the parasite threshold for RDTs is higher at 200 parasites per uL compared to that of microscopy at 15 parasites per uL, RDTs do not need trained technicians with appropriate equipment.[3,5] RDTs detect parasite antigens in the blood using specific monoclonal antibodies through immunochromatographic approaches.[13,36] These RDTs can detect soluble *Plasmodium* antigens including histidine-rich protein 2 (HRP2), adolase, and *Plasmodium falciparum* lactate dehydrogenase (pfldh).[13,36] Because RDTs detect circulating parasite antigens such as HRP2 even when the parasite is sequestered in the placenta, RDTs could be useful in diagnosing placental malaria in the absence of peripheral infection.[5,36,37]

The quality of the RDTs, especially HRP2-based tests, can be high in accuracy and stability.[36] However, potential limitations to the sensitivity of HRP2-based tests include antigenic variation or gene deletions of HRP2.[37] In addition, RDTs lack the sensitivity needed to detect the low-density peripheral parasitemia found in pregnant women.[3,47] Subpatent infections (i.e., parasitemia not detected by RDTs but detected through more sensitive methods) generally include submicroscopic infections and have been suggested by some researchers but not others to lead to negative birth outcomes.[4,5] RDT-based screening and treatment strategies that have been popularized recently thus fail to detect subpatent infections associated with placental malaria.[38] A

method to allow a more comprehensive understanding of the burden of malaria (both microscopic/patent and submicroscopic/subpatent infections) that is not readily available in low and middle-income countries is molecular detection methods such as PCR.[38,47]

Measuring peripheral parasitemia using polymerase chain reaction

An alternative method for diagnosing malaria infection is PCR which detects nucleic acid.[36] PCR is the most sensitive detection method of malaria (both peripheral and placental malaria) and has allowed studies of low-density *P. falciparum* parasitemia, including studies determining malaria in pregnancy as pregnant women frequently have low levels of parasitemia.[36,41,47] Using standard PCR methods and genomic nucleic acid extracted from dried blood spots, researchers can detect more sensitive estimates of maternal malaria infection than microscopy or RDTs.[37,40,48,49] PCR can detect parasite densities as low as 1 parasites per uL of peripheral blood.[50] However, PCR is time consuming and requires specialized laboratories and trained staff, and therefore is not available for routine diagnosis in resource-limited areas.[36,37,41]

PCR is able to detect at very low levels parasite nucleic acids, but these nucleic acids could be residuals from a viable parasite or gametocyte or a non-viable sequestered parasite.[36] In addition, PCR has been shown to have similar difficulty in diagnosing active placental infections during pregnancy as other methods that use peripheral blood. Use of peripheral blood PCR to diagnose placental malaria failed to detect more than half of the active placental infections in one study, likely because of sequestration of *P. falciparum* parasites in the placenta and evasion of spleen clearance in circulation.[5,35,43] However, other reports found higher sensitivities of placental malaria

using peripheral blood PCR.[43] In addition, as placental infections cannot be determined until after delivery, the best approximation is using peripheral blood to detect malaria infection.[36,37,43] Finally, PCR is able to detect parasitemia below the thresholds of microscopy and RDTs, but the clinical relevance of these submicroscopic/subpatent infections are uncertain.[36,45]

Measuring peripheral parasitemia using polymerase chain reaction: subpatent and submicroscopic infections

Subpatent infections are defined as peripheral blood infections below the detection limit of RDTs, but are detected through more sensitive methods such as PCR.[41] Subpatent infections are very common in areas of high malaria transmission.[41] In all malaria transmission settings, adults have the highest proportion of subpatent infections.[49]

The health consequences of subpatent infections are unclear.[41,45] Subpatent infections have been associated with poor maternal and birth outcomes including maternal anemia in pregnancy and lower birth weight.[45] However, in a study among pregnant women living in an area of high malaria transmission in Malawi, untreated subpatent infections were not associated with negative health outcomes for the mother or child.[45] Researchers have suggested that these higher-density subpatent infections may bolster the maternal immunity response to parasites that sequester in the placenta, mitigating adverse outcomes.[45]

Submicroscopic infections cannot be identified through microscopic examination of a blood smear, and can only be identified through more sensitive methods including molecular detection.[38] Submicroscopic peripheral malaria infections are defined as peripheral blood parasites detected through more sensitive methods such as PCR but

not through blood smear.[38] Submicroscopic placental malaria infections are defined as placental parasites detected through more sensitive methods but not through histology.[38] RDTs generally have a higher limit of detection of parasites (about 200 parasites per uL of peripheral blood) compared to microscopy (generally lower limit of about 40-50 parasites per uL of peripheral blood, can be as low as 15 parasites per uL)[3,45]; thus by parasite threshold, subpatent infections generally include submicroscopic infections.

Submicroscopic malaria infections may be significant contributors to malaria transmission, and may predominate in low malaria transmission settings.[49] The prevalence of submicroscopic infections varies depending on transmission intensity, and in a wide range of transmission settings, submicroscopic infections in pregnancy have been shown to be several times more common than microscopic infections in pregnancy.[38,49] In some settings, prevalence of submicroscopic malaria infections in pregnancy has been as high as 55%.[51]

Submicroscopic infections are likely more clinically relevant during pregnancy, as any density of malaria parasitemia could lead to negative health outcomes for the mother and her fetus.[51] However, the clinical significance of these submicroscopic infections is still a matter of some debate.[36,45] First, as PCR measures parasite nucleic acids, use of PCR to diagnose placental malaria could instead be detecting non-viable sequestered parasites.[36] In addition, pregnant women can spontaneously clear submicroscopic infections.[38] Some researchers have suggested that host immunity inhibits parasites to these low-level densities, and that women who are able to control their parasite density to submicroscopic levels may not experience negative birth

outcomes associated with placental malaria infections.[43,51] In contrast, other studies have found that women who have submicroscopic malaria infections have a higher risk of maternal anemia and low birth weight compared to women with no infections, but a lower risk when compared to women with microscopic malaria infections.[17,36–38] In addition, submicroscopic malaria infections detected at delivery increased the risk of stillbirth.[52] These low-density infections could be recently acquired infections that if left untreated, could reach microscopic-detectable densities and lead to more serious negative health outcomes.[36,51]

Because submicroscopic infections are undetectable through standard techniques and are often asymptomatic, they are frequently untreated through routine ANC despite potentially leading to negative maternal and infant health outcomes.[36,51,52] Among primigravidae, submicroscopic infections but not microscopic infections were associated with higher risk of low birth weight, potentially because microscopic infections were immediately treated while submicroscopic infections were not.[4] These untreated and prolonged submicroscopic infections consequently can lead to negative health outcomes.[4,38]

Women who are infected with malaria before pregnancy are more likely to be infected while pregnant, suggesting that some of the malaria infections detected during pregnancy were acquired before pregnancy.[21,53] These malaria infections include infections undetectable through microscopy and can persist through conception until the first ANC visit when they are treated through IPT-SP.[38,53] These untreated infections, including those that are spontaneously cleared before the first ANC visit, may cause placental pathology.[38]

Submicroscopic infections are very common at the first ANC visit, suggesting a need for earlier treatment and protection from malaria in order to prevent malaria in pregnancy.[38] This burden of submicroscopic or subpatent malaria infection particularly affects women in their first trimester of pregnancy as they are excluded from mass drug administration programs and are often treated based on the RDT malaria result.[41] Therefore, these undetected and untreated infections among women in their first trimester of pregnancy can support malaria transmission and undermine malaria elimination strategies.[41] Many malaria interventions such as distribution of ITNs and IPT-SP are carried out once women seek ANC which is typically in the second or third trimester.[38] However, by this point, peripheral and placental infections can already be established leading to negative birth outcomes.[38] We plan to determine if peripheral infections detected in the first trimester lead to negative maternal and birth outcomes.

Protective measures of malaria in pregnancy

To prevent malaria in pregnancy, the WHO recommends prompt diagnosis and treatment of malaria, IPT-SP and ITN/LLINs to prevent malaria in pregnancy.[2] However, these strategies are challenging to initiate for women early in their pregnancy and habitually leave women unprotected from malaria in the first trimester.

Diagnosing malaria in pregnancy is difficult as pregnant women frequently have low levels of parasitemia that are asymptomatic and are below the detection limit of common diagnostic tools such as microscopy and RDTs.[3–6] These submicroscopic or subpatent malaria infections are found frequently in women in their first trimester of pregnancy, and may be acquired before conception.[8,21,53] As treatment of women in early pregnancy is based on these RDT malaria results, pregnant women are not

treated for these potentially undetectable infections.[41] Intermittent preventive therapy with sulfadoxine-pyrimethamine (IPT-SP) can clear these submicroscopic infections, but is contraindicated during the first trimester because of potential teratogenic effects; thus, women with submicroscopic or subpatent infections in their first trimester are unprotected from these potentially asymptomatic infections that can still cause placental pathology and negative birth outcomes.[7,23,38,54]

The WHO recommends the use of IPT-SP as early as possible in the second trimester, and at each scheduled ANC visit at least one month apart.[13] SP clears both existing drug-sensitive parasites and prevents incident infections.[40] However, SP is contraindicated during the first trimester because of potential teratogenic effects.[7,23,54] Specifically, SP is a folate antagonist which is associated with neural tube defects, and as neural tube closes during the first trimester, SP is contraindicated during this time.[7] While use of SP only after the first trimester minimizes the risk of teratogenicity, it means that malaria infection during trophoblast invasion or placentation is not addressed by IPT-SP.[55] Therefore, women in their first trimester are still vulnerable to malaria infection obtained during early pregnancy or prior to conception despite use of IPT-SP.

ITNs can prevent malaria infection during all stages of pregnancy, including this vulnerable time of preconception and early pregnancy.[56] ITNs can substantially reduce the risk of malaria in pregnancy, and use of ITNs have been shown to reduce low birthweight, miscarriage, stillbirths, placental parasitemia, and neonatal and child mortality.[56,57] They are the most well-known and preferred method to prevent malaria in pregnancy.[56] However, pregnant women frequently do not use ITNs.[56] In 2010, about

40% of pregnant women in sub-Saharan Africa slept under an ITN, and less than 60% of pregnant women who own an ITN use them regularly.[8,58] Many pregnant women in malaria-endemic settings do not use ITNs because of discomfort associated with sleeping under the ITN, insufficient motivation to use the ITN, and lack of ownership of ITNs.[3–8] In addition, women before and during their first pregnancy use ITNs substantially lower than women of higher parities; the median estimate of ITN use immediately prior to the first pregnancy was 25% while prior to subsequent pregnancies was above 40%.[8] Many pregnant women obtain their first ITNs at their first ANC visit which often occurs in the second trimester, and thus are not protected prior to their first ANC visit in their first pregnancy (i.e., during their first trimester of their first pregnancy).[21,23] Overall then, nulliparous women are rarely protected by ITNs prior to or during their first trimester of pregnancy.

These current preventative strategies of diagnosing and treating, IPT-SP, and ITNs are frequently not used during the first trimester, leaving women unprotected from malaria during this period. In addition to protective measures, malaria in pregnancy is impacted by transmission intensity and the immunity of the mother.

Impact of transmission intensity and immunity on malaria

Malaria transmission intensity has serious implications on the acquisition of natural immunity to malaria.[59] Through repeated exposure to the *P. falciparum* parasite, individuals in moderate to high malaria transmission areas develop protection against malaria.[26,60] With subsequent exposures, they build immunity, first to severe malaria, then illness with malaria, and ultimately immunity against microscopic-detectable parasitemia.[26,60] This immunity is associated with antibodies developed from previous

exposure against different parasite isolates and reduces the frequency and density of parasitemia.[44] As a consequence, in areas of high malaria transmission, morbidity and mortality from malaria is highest in early childhood and asymptomatic malaria is common in older children and adults in high transmission areas.[26,60]

Individuals in low, unstable, or seasonal malaria transmission areas have infrequent exposure to infection, and therefore have low immunity.[61] Without this acquired protection, infection with malaria often leads to symptomatic disease and can progress to severe malaria with serious negative health outcomes regardless of age.[13,26,61] However, in all transmission settings, women who are pregnant are at higher risk of malaria infection.[44]

Impact of pregnancy on risk of malaria

The susceptibility of women to malaria infection increases in pregnancy because immunomodulation occurs during pregnancy as the immune system changes to accept the fetal allograft.[44] This increased susceptibility is partially due to the *P. falciparum* parasite avoiding splenic clearance and sequestration in the placenta.[41,62,63] Infection during pregnancy also decreases the level of specific antibodies, increasing the risk of complications due to malaria in pregnancy.[64]

Even in areas of high malaria transmission where pregnant women have acquired a partial immunity to malaria, the risk of malaria infection increases when a woman becomes pregnant.[41,59,65] Pregnant women from all transmission settings frequently experience asymptomatic peripheral parasitemia and are at much higher risk of severe disease compared to non-pregnant women.[59,65] However, even in the absence of documented peripheral parasitemia and asymptomatic infection, the

placenta can be heavily infected with *P. falciparum* parasites, consequently leading to reduced birthweight.[2,66–68] The risk of delivering a low birthweight infant doubles when women have placental malaria infection.[1,26] Asymptomatic malaria in pregnancy can also lead to severe maternal anemia and infant mortality.[1,13,26,69]

The intensity of malaria transmission likely affects the relationship between maternal antibody responses and malaria in pregnancy.[70,71] Pregnant women living in areas of moderate to high malaria transmission may develop broader antibody responses to *P. falciparum* infection earlier in pregnancy compared to pregnant women living in areas of low or seasonal malaria transmission.[70,71]

Most research on malaria in pregnancy is from areas of high transmission, and a review of the literature from 1985 to 2000 found the prevalence of malaria in pregnancy ranged from 10-65% among all gravidae and a median prevalence of 27.8%.[1,63,72] As the transmission intensity of malaria decreases, the prevalence of malaria in pregnancy also decreases.[13,73] However, in areas of lower transmission intensity, the malaria-specific antibodies also wane, leading to increased parasite burden and negative health outcomes during any malaria infection.[13,73]

In these low or unstable malaria transmission areas, malaria in pregnancy is still a large burden and the estimated prevalence of peripheral malaria infection was 13.7%, and the median placental malaria prevalence was 6.7%.[1,63] Pregnant women with malaria in low transmission areas are often symptomatic as they have not developed the partial immunity to malaria, and thus suffer from fever, abdominal pain, headache, vomiting, and nausea.[59,67] These pregnant women are also at higher risk of developing severe malaria.[26] Pregnancy complications including spontaneous abortions, fetal

distress, preterm birth, stillbirth, and low birth weight frequently occur among women infected with malaria in pregnancy who also live in areas of low transmission.[26,59,69,74]

The relationship between malaria morbidity and level of transmission is of increasing importance as malaria transmission decreases globally.[46] However, even though malaria in pregnancy appears to have different health implications depending on the level of malaria transmission in the community, the overall disease burden could be similarly heavy without preventive measures including those targeted towards women with the highest risk of malaria in pregnancy.[13]

Impact of immunity on malaria in pregnancy

All parity groups have an increased susceptibility to malaria infection in pregnancy.[13,67] However, over successive pregnancies, women naturally acquire resistance to malaria in pregnancy.[13] *P. falciparum*-infected erythrocytes bind to the syncytiotrophoblast, and this sequestration in the placenta is mediated by VAR2CSA.[12] *P. falciparum*-infected erythrocytes avoid splenic clearance by binding to CSA in the placenta.[13] Over successive pregnancies, antibodies to VAR2CSA are developed and thus inhibit binding of *P. falciparum*-infected erythrocytes to the placenta.[12] This parity-dependent malaria immunity is associated with reduced parasite density, decreased clinical disease, and reductions in placental and peripheral parasitemia.[13]

Therefore, women without this acquired parity protection are at greatest risk to malaria in pregnancy and consequent negative health outcomes.[13] Primigravidae women have not yet acquired this pregnancy-specific immunity of antibodies against VAR2CSA, and consequently cannot prevent infected erythrocytes from binding to CSA in the placenta or clear placental parasites quickly and thus suffer from chronic

placental infection which is associated with low birth weight and maternal anemia.[13–17] The increased prevalence and parasite density of *P. falciparum* among primigravidae during pregnancy is found at all levels of malaria transmission.[44]

In areas of low transmission, the difference in parity-dependent susceptibility is less marked although primigravidae still have a higher parasite prevalence.[59] In high malaria transmission areas, the acquired immunity from stable exposure to *P. falciparum* parasites frequently leads to asymptomatic malaria even though the placenta may be heavily infected leading to consequent negative birth outcomes.[66,67] This parity-dependent susceptibility places primigravidae women at greatest risk to high parasite density and negative health outcomes.[13]

The prevalence of malaria in primigravidae may be high because of infected women becoming pregnant, rather than pregnant women becoming infected.[21] These long-duration infections acquired pre-conception may be frequent as ITN use is low among young women.[21,75,76] Many women obtain ITNs through ANC visits, and thus are not protected in their first pregnancy before their ANC visit.[21] If these ITNs are retained and used, women in subsequent pregnancies will be more protected from malaria infection in pregnancy and subsequent health outcomes.[21] However, this distribution of ITNs at ANC leaves primigravidae women, who are already at higher risk of malaria infection because of parity-dependent immunity, unprotected until their first ANC.[21]

Potential biological mechanisms of malaria in the first trimester

Pregnant women are at increased susceptibility to malaria partially due to immune system changes in order to accept the fetal allograft, and partially due to the *P. falciparum* parasite avoiding spleen clearance and accumulating in the placenta.[41,44,62,63]

Sequestration of malaria in the placenta and consequent increase in the risk of low birth weight has been established.[1] Low birth weight caused by malaria is likely through intrauterine growth restriction and preterm delivery.[77] Parasite sequestration can cause placental insufficiency, leading to decreased nutrient transport that likely leads to intrauterine growth restriction.[55] Preterm delivery also contributes to low birth weight and has been associated with malaria infection near delivery.[1]

Malaria infection in the first trimester could impact placental development and thus affect fetal growth.[55,68,78] Placental circulation development occurs during the first trimester, peaking between 10 to 12 weeks of gestation.[79] In addition, *P. falciparum* parasitemia is highest between 13 to 18 weeks of gestation.[66] As the peak prevalence of malaria in pregnancy is in late first trimester and early second trimester, the susceptibility to malaria must increase in the first trimester.[1] Changes in splenic function caused by pregnancy can increase the risk of *P. falciparum* infection in primigravidae as early as 8 weeks of gestation.[1,80]

While the impact of malaria during embryogenesis (which occurs between 6 to 13 weeks of gestation) when erythroblasts are the primary form of circulating red blood cells is not fully understood, current evidence suggests that negative birth outcomes are correlated with placental sequestration of erythrocytes and that malaria infection alters placental vascular development.[7,54,62,63,69]

P. falciparum-infected red blood cells bind to the syncytiotrophoblast in the placenta, a process mediated by VAR2CSA.[12] This sequestration of malaria parasites is associated with fetal growth restriction.[10] However, sequestration has been suggested to not occur in the first trimester as infected red blood cells cannot adhere prior to the

formation of the placenta which occurs approximately at the 14th week of gestation.[78] The fetus develops in a hypoxic medium prior to the vascularization of the intervillous spaces around the 12th week of gestation.[62] Therefore, infected erythrocytes from maternal blood should not have access to the intervillous spaces of the placenta and thus theoretically be unable to adhere to CSA expressed in these spaces.[62] Thus, the infections occurring in early pregnancy were suggested to only induce negative birth outcomes by occurring after (or persisting until after) the placenta is formed, and then cytoadhering to the placenta.[78] However, Doritchamou et al. found that *P. falciparum* parasites isolated as early as 7 weeks of gestation transcribed VAR2CSA and exhibited CSA-adhering phenotypes, thus suggesting the possibility of sequestration this early in pregnancy.[62] Our understanding of placental vascularization may be incomplete, as intervillous blood flow has been reported from the 6th week of gestation and thus placental irrigation may develop earlier than previously concluded.[62] In addition, the trophoblasts from the fetus that invade maternal uterine arteries may express CSA, thus potentially providing infected erythrocytes an initial location for sequestration during the first trimester.[62] Therefore, women in the first trimester may be infected with malaria parasites that express VAR2CSA, and thus could potentially have sequestered pregnancy-associated parasites leading to negative birth outcomes.[62]

Alternatively, malaria infection in the first trimester could impact the placentation process and thus impact fetal growth.[55,68,78] During this time, the trophoblast invades and remodels the maternal uterine arteries in order to increase uterine artery blood flow.[48] Trophoblast invasion is essential for normal placental function and fetal growth, and occurs from very early in pregnancy until 18-20 weeks of gestation.[10] However,

placentation is particularly sensitive to pathology in the first trimester, and malaria in early pregnancy can inhibit trophoblast invasion.[9,10] Disruptions can impair placental function by reducing utero-placental blood flow contributing to the pathogenesis of low birth weight and intrauterine growth restriction.[9,10,48]

Research has suggested that malaria in early pregnancy may lead to disruptions in placentation. For instance, plasma collected from pregnant women infected with *P. falciparum* malaria between 16 and 22 weeks of gestational age was shown to inhibit trophoblast migration but not trophoblast viability.[81] A small study of 4 pregnant women found that malaria infection before 24 weeks of gestation is associated with smaller placental volume which is linked to impaired trophoblast invasion.[82] Griffin et al. found that malaria infections occurring prior to 20 weeks of gestation affects uterine and umbilical artery blood flow.[48] Among primigravidae, malaria infection prior to 20 weeks of gestation decreased umbilical artery resistance in the third trimester, suggesting adaptive villous angiogenesis and increased risk of intrauterine growth restriction.[48] In contrast, McGready et al. found that malaria detected and treated early in pregnancy in an area of low transmission rarely leads to placental changes indicating potential placental recovery.[10,83] However, these studies all used wide intervals to define early pregnancy.

Using a narrow interval, Moeller et al. found that malaria in the first trimester (i.e., before 15 weeks of gestation) was associated with negative impacts on placental vascular development and consequent pregnancy outcomes.[11,12] However, this relatively small study of 68 women had recruited mostly multigravidae in an area of Tanzania with decreasing malaria transmission.[11,12] Changes in placental vascularity

would be even more pronounced among primigravidae or nulliparous women, and among women living in high malaria transmission areas.[11]

Although the first trimester might represent a critical time for intervention to prevent the negative consequences of malaria in pregnancy, few studies have assessed malaria in the first trimester because most studies are limited to observations in the second trimester when women begin receiving ANC.[23,77] In addition, studies of pregnant women in the first trimester are faced with serious challenges including time-consuming and expensive longitudinal follow-up and difficulties obtaining accurate measurement of gestational age.[23,77]

Previous research on malaria in early pregnancy

Research of low *P. falciparum* transmission settings and impact on early pregnancy are restricted to a few studies. In 1989 to 1990, a case-control and community cohort study examined the association of malaria infection and low birth weight in Sudan in an area of low to moderate transmission, and found that the risk of low birth weight was highest if the malaria infection occurred in the first trimester.[84] In addition, women in their first pregnancy had a high risk of low birth weight.[84] This study relied on retrospective malaria history during pregnancy given by the mother at delivery, potentially leading to differential recall bias and misclassification.[39,84]

Most research on low malaria transmission settings in early pregnancy is based at the Shoklo Malaria Research Unit located at the Thai-Burmese border.[10,52,54,82,85] This southeastern Asian cohort with both *P. falciparum* and *P. vivax* malaria transmission began in 1986 and has collected data for over 30 years.[10,52,54,82,85] Although exposure to two *Plasmodium* species, and changes in malaria transmission over time, methods

used to measure gestation age, and quality of ANC could have affected their results, they found that a single episode of *P. falciparum* malaria in the first trimester (< 14 weeks of gestation) was associated with miscarriage, especially among those with two or more infections in the first trimester.[52,54,85] However, a successfully treated episode of *P. falciparum* malaria in the first trimester was not associated with low birth weight, suggesting either minimal impact of the malaria infection in the first trimester on birth weight or placental recovery *in utero*.[85] The rates of *P. falciparum* malaria were highest at 6 weeks of gestation and declined as the pregnancy progressed, and symptomatic or asymptomatic *P. falciparum* malaria detected and treated after 12 weeks of pregnancy was associated with increased risk of small for gestational age, but they found no association if the malaria infection was detected and treated between 4 to 12 weeks of gestation.[10] However, this southeastern Asian cohort with low transmission of both *P. falciparum* and *P. vivax* may not be representative of the malaria burden of women in sub-Saharan Africa where *P. falciparum* substantially predominates or in areas of high transmission.[52]

Several more studies have focused on malaria infection in early pregnancy in higher *P. falciparum* transmission settings in sub-Saharan Africa. Cottrell et al. conducted a retrospective analysis of pregnant women from an area with high malaria transmission burden in Burkina Faso from 1987-88 and found a decreasing trend in the mean birth weight when peripheral malaria infection was detected before 4 months of gestation.[39] Griffin et al. studied the impact of early malaria infection on fetal growth and uterine growth in a small ultrasound study of 128 pregnant women from the DRC from 2005-06.[48] They found that malaria infection before 20 weeks of gestation is associated

with intrauterine growth restriction among primigravidae, and decreased uterine and umbilical artery blood flow in the late third trimester.[48] Valea et al. studied pregnant women in Burkina Faso from 2006-08, and found that the 31 women studied who were infected with malaria during the first trimester had higher risk of delivering a low birth weight baby compared to women who were never infected with malaria during pregnancy.[86] In contrast, De Beaudrap et al. found no association between a single detected and treated infection before 20 weeks of gestation and low birth weight.[6,47] However, this study of pregnant Ugandan women conducted between 2006-09 had only 12 women infected with malaria prior to 15 weeks of gestation out of the total 120 pregnant women recruited by that time of gestation, and therefore the impact of malaria in the first trimester could not be accurately assessed.[6,47] Kalilani-Phiri et al. determined that among a cohort of Malawian pregnant women studied between 2009-10, infections that occurred in early pregnancy prior to or at the onset of the first ANC visit was associated with placental infection, but discarded the 36 pregnant women recruited in the first trimester from their analysis because of small numbers.[87]

Ancillary studies on the STOPPAM (Strategies to Prevent Pregnancy-Associated Malaria) prospective cohort conducted in Tanzania and Benin in 2008-11 found that Tanzanian pregnant women infected within the first 4 months of pregnancy had restricted fetal growth among primigravidae, restricted intrauterine growth in the third trimester, reduced birth weight, and reduced placental weight.[9,88] When restricting their study population to women recruited before gestational day 97 (i.e., first trimester), their analysis of 14 exposed and 46 non-exposed women found a non-significant reduction in mean birth weight among women exposed to malaria in the first trimester.[88] In Benin,

Briand et al. used the same study design (as Schmiegelow et al. did in Tanzania), and found that infections in early pregnancy were associated with reduction in fetal growth velocity.[9,89] Also in Benin, Huynh et al. found that malaria infection prior to the fifth month of gestation was associated with decreased mean birth weight and increased maternal anemia.[78] Finally, Cottrell et al. found that submicroscopic infections early in pregnancy among Beninese women (i.e., at inclusion - mean gestational age 16.5 weeks, standard deviation 4.8 weeks) were associated with an increased risk of low birth weight among primigravidae.[4]

Elphinstone et al. found that among a cohort of Malawian women enrolled between 2011-13, malaria infection detected before 24 weeks of gestation was associated with an increased risk of preterm birth.[7] However, they only enrolled women were had at least 13 weeks of gestation, and therefore their analysis did not include most of the first trimester of pregnancy.[7] Moeller et al. examined 28 placentas infected before 15 weeks of gestation, and found that malaria before 15 weeks of gestation negatively impacted placental vascular development.[11]

In addition, other studies have examined malaria in early pregnancy but have not focused on birth outcomes. Berry et al. determined risk factors of malaria in early pregnancy in areas with seasonal malaria transmission by using data from a randomized control trial conducted in Burkina Faso, The Gambia, Ghana and Mali.[21] They found that risk factors of malaria infection at the first ANC visit (between 16 and 30 weeks of gestation) include the duration of pregnancy spent in the rainy season, age, gravity, and socioeconomic status, but did not find an effect of bed net use on malaria infection at enrollment.[21] In addition, while Kapisi et al. recruited women between 12 to

20 weeks of gestation, they did not examine the relationship between malaria in early pregnancy and adverse birth outcomes but instead examined the burden of malaria during pregnancy (i.e., number of infections during pregnancy) and adverse birth outcomes.[90]

The above studies' limitations include data collection occurring decades ago, small numbers of pregnant women in early pregnancy examined, large early pregnancy intervals (e.g., first half of pregnancy, less than 20 weeks of gestation), and imprecise measurements of gestational age. In order to address these limitations, the RECIPAL (REtard de Croissance Intra-uterine et PALudisme) preconceptional cohort was developed to assess the effects of malaria during the first trimester of pregnancy.[22,23,91–93] Briefly, from 2014-17, 1,214 Beninese women of reproductive age were recruited and followed until 411 became pregnant.[22,23,91–93] These 411 pregnant women were followed from 5-6 weeks of gestation (as determined by ultrasound) until delivery of which 273 women delivered with complete follow-up.[22,23,91–93]

These researchers found that the proportion infected with malaria (both microscopic and submicroscopic) was highest in the first trimester and that malaria infection in the first trimester was associated with an increased risk of maternal anemia in the third trimester, but was not associated with an increased risk for negative birth outcomes including preterm birth, small for gestational age, or low birth weight.[23,92] In addition, ITN use in the first trimester reduced the risk of malaria infection, and women infected before conception were more likely to be infected in the first trimester.[22,91,93] While the RECIPAL study has many strengths including screening of women very early in the first trimester and accurate gestational age dating though ultrasounds, the cohort

included only 24 births with complete follow-up from primigravidae even though primigravidae are the most likely to have negative birth outcomes due to malaria infection.[92] Therefore, the prevalence of negative birth outcomes was likely underestimated by the underrepresentation of primigravidae in the final RECIPAL cohort.[91]

These studies suggest that malaria in early pregnancy, when current recommended strategies either cannot or are rarely used, is a critical under-protected period in pregnancy that potentially because of placental recovery is associated with some but not all poor health outcomes. However, these previous studies are limited by occurring decades prior, defining wide intervals for early pregnancy, using imprecise measurements of gestational age, examining only a single-site, and including small numbers of pregnant women and even fewer primigravidae women (who have the highest risk of malaria in pregnancy and are the least protected). In order to expand understanding of malaria in the first trimester, we have analyzed a large cohort of nulliparous women between 6-14 weeks of gestation (as determined by ultrasound) to characterize the predictors of malaria in the first trimester and estimate its effects on maternal and birth outcomes.

Predictors of malaria in pregnancy overall and in early pregnancy

Predictors assessed in this dissertation include maternal age, maternal height, maternal body-mass index (BMI), maternal education, seasonality, and socioeconomic status. We have summarized and compared the previous research on predictors of malaria in pregnancy overall or of malaria in early pregnancy in **Table** 2.2. The few studies focusing on predictors of malaria in early pregnancy have found conflicting

results on maternal age, socioeconomic status, and seasonality.[21,22,88] In addition, only one study examined maternal height, maternal BMI, or maternal education as a predictor of malaria in early pregnancy.[88] In contrast with the well-studied predictors of malaria in pregnancy, younger age was *not* conclusively associated with higher prevalence of malaria in early pregnancy.[7,20–23,43,57,94–101] In addition, while short stature in Malawi,[98] low BMI in Kenya,[99] and lower overall educational attainment in multiple countries[6,7,100–103] were associated with malaria in pregnancy overall, a single study did not find an association between maternal height, maternal BMI, and maternal education and malaria in early pregnancy.[88] We have addressed this clear gap in the knowledge base by characterizing predictors of malaria in the first trimester in a large multi-site study of nulliparous women.

Maternal and birth outcomes of malaria in pregnancy overall and in early pregnancy

Birth outcomes assessed in this dissertation include low birth weight, preterm birth, small for gestational age, and perinatal mortality. Malaria in pregnancy is associated with an increased risk of low birth weight, and in malaria-endemic areas, about one-fifth of cases of low birth weight is attributable to placental malaria infection.[25] Malaria in pregnancy causes low birth weight through preterm delivery and through intrauterine growth restriction.[25] Preterm delivery due to malaria infection may be caused by the response of the immune system prompting early delivery.[25] Intrauterine growth restriction due to malaria infection may be caused by decreased nutrient delivery to the fetus, leading to term small for gestational age birth.[25]

Maternal outcomes assessed in this dissertation include maternal anemia in late pregnancy and hypertensive disorders in pregnancy. Malaria in pregnancy overall is

associated with an increased risk of maternal anemia, with about one-fourth of severe anemia in pregnancy attributable to malaria infection.[25] Anemia in pregnancy due to malaria infection is caused by hemolysis, reduced red blood cell production, and increased splenic removal of red blood cells.[25] Hypertensive disorders in pregnancy including preeclampsia and gestational hypertension have been suggested to be associated with malaria in pregnancy by malaria parasites causing a dysfunctional placenta leading to hypertensive disorders.[104]

We have summarized the literature on the effect of malaria in pregnancy overall or of malaria in early pregnancy on maternal and birth outcomes in **Table** 2.3**.** The few studies have found conflicting results on the effect of malaria on preterm birth, maternal anemia, and low birth weight.[4,6,7,11,20,39,84–86,88,92] Three studies found no association between malaria in early pregnancy and small for gestational age,[7,10,92] and no studies have published on the effect of malaria in early pregnancy on hypertensive disorders or perinatal mortality. While malaria in pregnancy overall is associated with adverse outcomes including preterm birth, anemia in late pregnancy/at delivery, small for gestational age, and low birth weight, the few studies on malaria in early pregnancy show conflicting results. Therefore, we were able to address this need for further research by estimating the causal effect of malaria in the first trimester on maternal outcomes including anemia in late pregnancy and hypertensive disorders, and birth outcomes including preterm birth, small for gestational age, low birth weight, and perinatal mortality.

Table 2.2. Comparison of reported relationships of predictors to either malaria in pregnancy overall or malaria in early pregnancy

Predictor	Reported relationship(s) to:			
	Malaria in pregnancy (overall)		Malaria in early pregnancy	
	Higher prevalence of malaria	No association	Higher prevalence of malaria	No association
Maternal age (younger vs. older)	7,43,57,94,95,97–101,105	106,107	21	22,88
Maternal height (shorter vs. taller)	98	No evidence found	No evidence found	88
Maternal BMI (lower vs. higher)	7,99,108	109,110	No evidence found	88
Maternal education (lower vs. higher)	6,7,100–103	57,87	88	No evidence found
Seasonality (rainy vs. non-rainy)	95,98,106	53	21	22
Socioeconomic status (SES) (lower vs. higher)	7,94,103,111	108,110	21	22

Table 2.3: Comparison of reported relationships of either malaria in pregnancy overall or malaria in early pregnancy and maternal or birth outcome

Outcome	Reported relationship(s) to:			
	Malaria in pregnancy (overall)		Malaria in early pregnancy	
	Higher risk of outcome	No association	Higher risk of outcome	No association
Preterm birth	17,55,72,90,97,112–114	7,108	4,7	92
Small for gestational age	10,82,90,110	7,90	No evidence found	7,10,92
Low birth weight	7,16,72,89,97,113,115,116	108	4,11,20,39,84,86,88	6,85,92
Perinatal mortality	55,113,117–119	7,110,120	No evidence found	No evidence found
Anemia in late pregnancy/at delivery	16,17,97,105,112,121–123	87,124	4,20,88,92	86
Hypertensive disorders	125–128	125,126,129	No evidence found	No evidence found

CHAPTER III: METHODS

Study Site

The *Eunice Kennedy Shriver* NICHD Global Network for Women's and Children's Health Research was developed to conduct clinical trials in resource-limited countries to evaluate low-cost sustainable interventions aimed at improving health of women and children.[130] Since 2013, the NICHD Global Network has conducted research in multiple low and middle income countries, including three countries in sub-Saharan Africa: Democratic Republic of the Congo (DRC), Kenya, and Zambia.[130]

This malaria sub-study was nested within the NICHD Global Network's trial of low-dose aspirin for the prevention of preterm delivery in nulliparous women with a singleton pregnancy.[18,19] This Aspirin Supplementation for Pregnancy Indicated Risk reduction In Nulliparas (ASPIRIN) trial was a prospective, randomized, multi-national clinical trial that tested the hypothesis that low-dose acetylsalicylic acid initiated in the first trimester reduces the risk of preterm birth.[19] The trial recruited 11,920 nulliparous women between March 23, 2016 and April 11, 2019 at seven research sites in six countries (DRC, Zambia, Guatemala, Pakistan, Kenya, and two sites in India), who were randomly assigned 1:1 to receive either daily low-dose acetylsalicylic acid (81 mg dose) or a visually identical placebo, beginning in the first trimester (gestational age between 6 weeks, 0 days and 13 weeks, 6 days, confirmed by study ultrasound) and continuing until 36 weeks and 7 days of gestation or delivery.[19] Participants were

individually randomized by site and the randomization sequence was developed using a randomly permuted block design with varying block sizes.[19]

In the DRC, the Global Network sites are in the Northwest provinces of Nord and Sud Ubangi, and the site coordinating center is in Kinshasa.[130] There are 14 clusters which are each served by a health center where care is administered by nurses.[130] Physicians, nurse midwives, and nurses run the three hospitals; there are no specialty physicians available.[130]

In Kenya, the Global Network sites are in western Kenya in the counties of Busia, Bungoma, and Kakamega, and the site coordinating center is in Eldoret.[130] There are 16 clusters which are served by 23 health facilities where care is administered by nurse-midwives, clinical officers, and a single medical officer.[130] The three hospitals act as referral hospitals and are run mostly by generalist physicians, with a few trained obstetricians and pediatricians.[130]

In Zambia, the Global Network sites are in southern Zambia in the districts of Kafue and Chongwe, and the site coordinating center is in Lusaka.[130] There are 10 clusters, of which 8 have health posts where care is administered by nurse midwifes.[130] Traditional birth attendants provide care for home births.[130] In Lusaka, there are three district hospitals and a referral hospital, but specialty physicians are only available at the referral hospital.[130]

This malaria in the first trimester sub-study was conducted among 1,513 women in the ASPIRIN trial from the sub-Saharan African sites (485 women from DRC, 677 women from Kenya, and 351 women from Zambia (**Figure** 3.1). Women were recruited

from primary health-care centers and hospital-based clinics, and each site screened pregnant women residing within the community for eligibility in the ASPIRIN trial.[19]

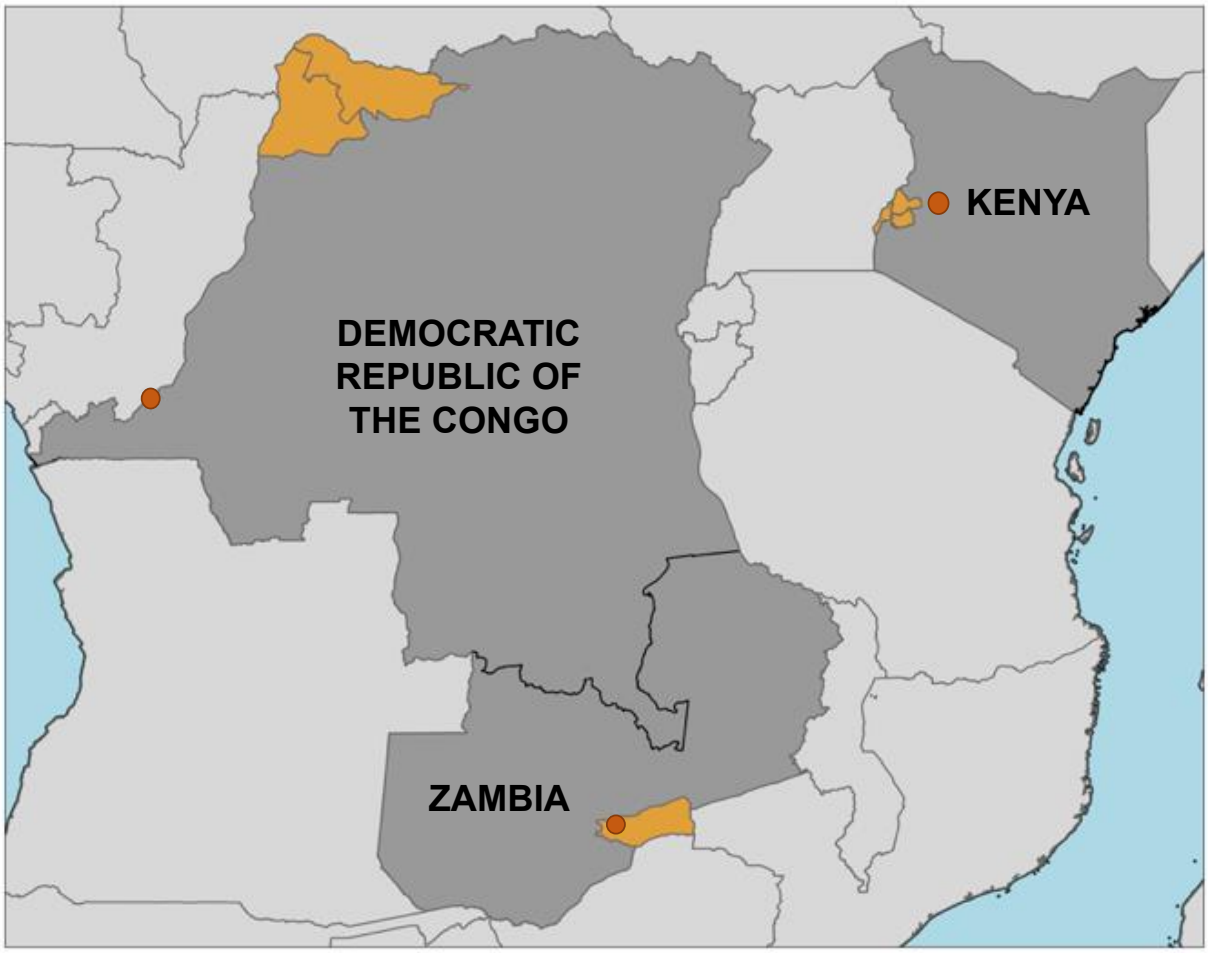


Figure 3.1: Map of NICHD Global Network sub-Saharan African sites
In the DRC, the research sites are in Nord and Sud Ubangi (shaded in light orange), and the site coordinating center is located in Kinshasa (the capital, noted by orange dot). In Kenya, the research sites are in the counties of Busia, Bungoma, and Kakamega (shaded in light orange), and the site coordinating center is located in Eldoret (noted by orange dot). In Zambia, the research sites are within the districts of Kafue and Chongwe (shaded in light orange), and the site coordinating center is located in Lusaka (the capital, noted by orange dot). Figure modified from Bose et al., 2015.[130]

Study Population

The ASPIRIN trial targeted nulliparous women who were in their first trimester of pregnancy from low and middle-income countries. The ASPIRIN trial recruited women

from six countries by using clinic-based and community-based recruitment strategies as determined by each study site in order to reach a diverse study population.[18,19] Women were asked if they were nulliparous and pregnant between 6 weeks 0 days and 13 weeks 6 days based on last menstrual period.[18] Ultrasound was used to determine gestational age after enrollment.[18] The source population for this sub-study were all pregnant women who participated in the ASPIRIN trial who lived in countries with endemic malaria (i.e., DRC, Kenya, and Zambia). The sampling population for this sub-study were pregnant women from DRC, Kenya, and Zambia who enrolled in the ASPIRIN trial and had dried blood spots (DBS) obtained at enrollment that were tested for malaria parasites and classified as positive or negative for malaria parasites.

The inclusion criteria from the ASPIRIN trial included nulliparous women between 18-40 years of age (possibility of minors who are 14 or older being enrolled if allowed by the country's guidelines) who are residents of the study area, who had no more than two previous first-trimester pregnancy losses and no medical contraindications to acetylsalicylic acid.[18,19] In addition, all women at enrollment must have had a single live intrauterine pregnancy that was between 6 weeks and 0 days to 13 weeks and 6 days in gestational age that was confirmed by an early dating ultrasound and the presence of a heartbeat.[18,19]

Exclusion criteria for the ASPIRIN trial included women who were already prescribed daily acetylsalicylic acid for more than a week, and women with multiple gestations.[18,19] Further exclusion criteria included a fetal anomaly detected by ultrasound at screening, women with severe anemia (hemoglobin levels < 7.0 g/dL at screening), women with hypertensive disorders (systolic blood pressure ≥ 140 mm Hg

and diastolic ≥ 90 mm Hg at screening), or any women with any medical condition that may be a contradiction to receiving acetylsalicylic acid, as evaluated by the site investigator (e.g., Type 1 diabetes, lupus, hypertension, or other significant disease).[18,19]

The inclusion criteria for the first aim assessing predictors of malaria in the first trimester included all randomized participants from the DRC, Kenya, and Zambia who had a DBS collected in the first trimester and a quantitative PCR (qPCR) malaria result of positive or negative (n=1513). For the second aim estimating effects on maternal and birth outcomes, participants additionally had to provide any post-baseline outcome data and have delivered at 20 weeks of gestational age or greater (n=1446).

Participant Data and Sample Collection

At enrollment, information was collected on demographics (including years of maternal age and education), pregnancy and medical history, and current medical information (including height in centimeters, weight in kilograms, blood pressure, heart rate, and history of diabetes).[19] Dried blood spots (DBS) were also collected by pricking the participant's finger and placing three blood spots on filter paper, which were then completely dried before storage at room temperature in plastic bags with desiccant. DBS were also collected in late pregnancy between 26-30 weeks of gestation. DBS were collected from all eligible women who consented to this sub-study who were enrolled between January 2016 to April 2018.

Maternal outcomes included vaginal bleeding, antepartum hemorrhage, postpartum hemorrhage, maternal mortality, late pregnancy termination, change in maternal hemoglobin, and preeclampsia and eclampsia.[19] Fetal and newborn outcomes

included preterm birth, low birth weight, fetal loss, spontaneous abortion, stillbirth, small for gestational age newborn, perinatal mortality, and medical termination of pregnancy.[19] Maternal and birth outcomes were obtained up to 42 days following delivery using the Global Network Maternal and Newborn Health Registry.[19]

Sample Processing

Participant DBS samples were shipped to the Meshnick lab at the University of North Carolina at Chapel Hill in Chapel Hill, North Carolina where they were processed to detect *P. falciparum*. DBS were retrospectively tested in duplicate for *P. falciparum* lactate dehydrogenase (pfldh) nucleic acid using qPCR, a sensitive molecular detection method.[131] DBS were distributed into a 96-well plate with one punched DBS per participant per well. *P. falciparum* parasite nucleic acid was extracted from each sample using Chelex. Each sample was tested in duplicate for pfldh nucleic acid using qPCR; human ß-tubulin was used as a nucleic acid control. Samples were defined as positive for *P. falciparum* infection when florescence for both replicates crossed the threshold prior to the 39th cycle, or when one replicate did not amplify and the other crossed the threshold prior to the 39th cycle. Discordant results between duplicates were excluded from analysis. A convenience sample of DBS was included in this analysis.

Defining the analytic data set

Of the 3,628 dried blood spots analyzed by qPCR, 3,497 were successfully merged with the ASPIRIN dataset, had a non-missing expected delivery date, and had a non-missing collection date. The difference between the expected delivery date and the collection date was determined and subtracted from 280 days (i.e., 40 weeks) to obtain the estimated gestational age at sample collection in days. This estimated gestational

age at sample collection in days was then divided by 7, and then rounded to the floor to obtain the estimated gestational age at sample collection in weeks (i.e., 6 weeks 6 days became 6 weeks). The first trimester was defined as having an estimated gestational age between 6 weeks 0 days and 13 weeks 6 days inclusive at the time of collection.

In order to ensure that only one sample from each woman would be defined as obtained in the first trimester, the de-duplication process varied depending on gestational age at each sample collection and the qPCR result of the sample. If multiple samples from the same woman were defined as obtained in the first trimester and had different gestational age in weeks at sample collection, the sample collected at the earlier gestational age was included. De-duplicating samples was based solely on which sample had the earlier gestational age at collection in weeks as the qPCR result of positive or negative for *P. falciparum* was not examined. If multiple samples from the same woman were defined as obtained in the first trimester and had the same gestational age in weeks at sample collection, de-duplication then depended on the qPCR result for *P. falciparum.* If the results were concordant, then one sample was randomly labeled as a duplicate. If one result was positive or negative and the result was not available (i.e., not positive or negative), the sample with a positive or negative result was included and the sample with a not available result was excluded. Finally, if results were discordant, all samples were labeled as discordant and excluded from further analysis. After de-duplication, any sample that was included but not randomized in the ASPIRIN trial was excluded.

Thus, only non-duplicated randomized samples classified as obtained in the first trimester from DRC, Kenya, and Zambia with a qPCR result of positive or negative for

P. falciparum infection were included. Thus, analysis on predictors of malaria in the first trimester is based on 1,513 samples each collected from a nulliparous woman in the first trimester of pregnancy from DRC, Kenya, and Zambia. As participants additionally had to provide any post-baseline outcome data and have delivered at 20 weeks of gestational age or greater to be included in the study population for Aim 2, analysis of the effect of malaria in the first trimester on maternal and birth outcomes is based on 1,446 samples each collected from a nulliparous woman in the first trimester from DRC, Kenya, and Zambia.

Innovation and comparison to previous studies

To clarify what increases the likelihood of malaria in the first trimester, and to estimate the causal effects of malaria in the first trimester on adverse maternal and birth outcomes, we used a stronger study design and improved methodology compared to previous work. We analyzed malaria in the first trimester among a large population of pregnant women from multiple sites compared to previous studies that were small and single-site. By including multiple sites, we were able to study how malaria morbidity changes in different transmission settings, which is of increasing importance as malaria transmission decreases globally.[46,125]

The women we studied were predominantly women with the highest risk of malaria in pregnancy and consequent negative health outcomes, i.e., women in their first pregnancy (primigravidae), compared to other studies that have mostly included multigravidae instead of primigravidae women.[13,44] Because they lack parity-dependent immunity that reduces placental and peripheral parasitemia, primigravidae women have the highest risk of malaria in pregnancy and consequent negative health outcomes.

Primigravidae have higher prevalence and parasite density of *P. falciparum* in pregnancy than women with higher parities.[44] As many pregnant women acquire ITNs through their first antenatal care visit, and women rarely use ITNs before and during their first pregnancy, this habitually leaves women in their first trimester of their first pregnancy unprotected from malaria infection.[8,21,23] While we studied nulliparous women, most women in our study were primigravidae, and thus our sub-study was able to address an important gap in knowledge about malaria in pregnancy among the most affected group.

We restricted our definition of early pregnancy to the first trimester (i.e., 6-<14 weeks of gestation) compared to previous studies that defined early pregnancy as wide as the first half of pregnancy. We used ultrasounds to accurately measure gestational age in the first trimester. Little is known about the biological mechanism of malaria in the first trimester. As the placenta forms about 14 weeks of gestation, whether sequestration of *P. falciparum* parasites in the placenta can occur in the first trimester is of some debate.[62,78] In addition, malaria infection before 15 weeks of gestation was associated with negative placental vascular development.[11] Therefore, by restricting malaria to the first trimester, we were able to estimate causal effects of malaria in the first trimester on adverse maternal and birth outcomes.

We used PCR, a very sensitive molecular detection method of malaria infection during pregnancy, compared to other studies that used placental histology, microscopy, or RDTs, that allows us to detect low-density infections common in pregnancy including subpatent and submicroscopic infections. The only way to detect malaria infection during pregnancy is through detection of parasites through peripheral blood.[36,37] As

parasitemia in pregnancy is often very low and undetectable by most standard methods, diagnosing malaria in pregnancy is difficult.[36,37] PCR is a very sensitive method to detect malaria infection in peripheral blood and can detect subpatent and submicroscopic infections.[37,40,48–50] By using a very sensitive method of detection of malaria infection, we were able to detect and analyze even low-level malaria infections in the first trimester.

We leveraged a past multi-national clinical trial that specifically recruited women in the first trimester (i.e., 6 weeks 0 days to 13 weeks 6 days of gestation) in order to decrease costs and increase efficiency while developing the first large multi-site study of malaria in the first trimester. Additional data collected for this sub-study were minimal (DBS at enrollment and at late pregnancy), and as low-dose aspirin was randomly assigned, we did not need to control for the effects of the ASPIRIN study protocol. From this DBS, we were able to determine *P. falciparum* infection at the first trimester of pregnancy including low-density infections that cannot be detected by RDT or microscopy, and then we linked these malaria test results to the extensive amount of already collected information. We thus were able to determine crucial information about predictors and consequent maternal and birth outcomes of malaria in the first trimester by leveraging an existing clinical trial.

In this first large multi-site study on malaria in the first trimester, we were able to characterize predictors of malaria in the first trimester and estimate the causal effect of malaria in the first trimester on adverse maternal and birth outcomes.

Data analysis

The data analysis approach was largely motivated to address current gaps in the

literature. As only two studies have assessed predictors of malaria in the first trimester,[22,88] we assessed six predictors to characterize the factors which are associated with increased likelihood of malaria in the first trimester. Likewise, limited research has assessed the impact of malaria in the first trimester on maternal and birth outcomes. The overall objective of this dissertation was to characterize predictors of malaria in the first trimester, and to estimate causal effects of malaria in the first trimester on adverse maternal and birth outcomes. All comparisons were limited to observations without missing data for each variable, and models were limited to observations without missing data for all covariates and the outcome. All analyses were conducted using R version 4.0.2.[126]

Aim 1 Predictor, Variable, and Outcome Assessment

The first specific aim was to characterize the factors which are associated with increased likelihood of malaria in the first trimester.

The main outcome was malaria in the first trimester, defined as a positive result for *P. falciparum* through qPCR in a sample obtained at enrollment.

Based on previous studies of malaria infection in late pregnancy, we examined the following predictors: maternal age, maternal height, maternal body-mass index (BMI), maternal education, season coincident with the first trimester, and socioeconomic status.

Maternal age was measured in years at enrollment. Maternal height was measured in centimeters at enrollment. Maternal BMI was calculated from the maternal weight recorded at enrollment in kilograms divided by the maternal height recorded at enrollment in meters squared. Maternal education was recorded at enrollment as no

formal schooling, primary education (1-6 years of schooling), secondary education (7-12 years of schooling), or university and beyond education (≥13 years of schooling). Season coincident with the first trimester of pregnancy was defined as rainy or not-rainy based on the month of the date of collection recorded on the malaria DBS filter paper sample with the specific months defined as rainy varying across countries: April to October in DRC,[133] April to June and October to November In Kenya,[134] and November to April in Zambia.[135]

To calculate socioeconomic status (SES), we used the Global Network Socioeconomic Status Index.[136] Developed from around 50,000 households of pregnant women included in GN, the Socioeconomic Status Index is calculated by determining the number of 10 specific items owned by the household to develop a country-specific score.[136] The specific items were dichotomized as yes or no. The sum score for Kenya or Zambia is the number of the following items that were owned by the household: improved source of drinking water, flush toilet, finished floor material, motorbike, car, smartphone, television, electricity, refrigerator, and use of liquid petroleum gas or electricity as cooking fuel.[136] Because few households in the DRC had a refrigerator or used liquid petroleum gas or electricity for cooking fuel, the SES Index instead uses two additional lower SES items for DRC: more than one room in home and bicycle.[136] Thus, the sum score for the DRC is the number of following items that were owned by the household: more than one room in home, bicycle, improved source of drinking water, flush toilet, finished floor material, motorbike, car, smartphone, television, and electricity.[136] From the sum score of the number of items owned, it was converted to an SES score specific for each country.[136]

Data on all predictors except season coincident with first trimester drew on the extant ASPIRIN study data which was reported by each of the sites to the Global Network Data Coordinating Center (DCC) (RTI International).[18] The data were used by the DCC to evaluate site performance including data quality.[18] To monitor data quality, a random 5% of the actual data collected was validated by chart review.[18] DCC staff conducted site visits as needed to review individual participant records and ensure compliance with the protocol and accuracy and completeness of the records.[18]

The last variable included was projected gestational age at enrollment which was developed from an algorithm described in Hoffman et al., 2020. Further details on the algorithm is presented in the Supplementary Information for Chapter IV on page 119.

Aim 1 Analysis

Based on previous studies of malaria infection in late pregnancy, we examined the following predictors: maternal age, maternal height, maternal body-mass index (BMI), maternal education, season coincident with the first trimester of pregnancy, and socioeconomic status. The ASPIRIN trial collected data required for all predictors at enrollment except for season coincident with the first trimester which was developed from the month of the collection data listed on the DBS assessed for malaria infection. The main outcome was malaria in the first trimester.

For continuous variables maternal age, maternal height, maternal BMI, and socioeconomic status, we created two categories by dividing at the country-specific median based on data from this sub-study. Maternal education was dichotomized into two categories: lower (i.e., no formal schooling and primary education (1-6 years of schooling)), and higher (i.e., secondary education (7-12 years of schooling) and

university and beyond education (≥13 years of schooling)). Season coincident with the first trimester of pregnancy was defined as rainy or not-rainy.

Crude prevalence ratios (PRs) and prevalence differences (PDs) were calculated for malaria in the first trimester by each variable, using stratified 2x2 tables for each country. We present 99% confidence intervals to account for multiple comparisons and limit inflation of non-coverage rates. Our parameters of interest were the crude (unadjusted) prevalence ratio and prevalence difference.

To assess heterogeneity by country, we calculated the I^2 value. If I^2 exceeded a pre-specified threshold of 40%,[137] we did not pool results across countries to calculate a summary estimate. If the I^2 value was ≤40%, we used the DerSimonian and Laird inverse variance method to calculate the summary estimate.[137]

Aim 2 Exposure, Confounder, and Outcome Assessment

The second specific aim was to estimate the causal effect of malaria in the first trimester on adverse maternal and birth outcomes. The main exposure was malaria in the first trimester, defined as a positive result for *P. falciparum* through qPCR in a sample obtained at enrollment.

To estimate the causal effect of malaria in the first trimester on the assessed maternal or birth outcome, we identified confounders of the exposure-outcome relationship using causal directed acyclic graphs (DAGs) based on prior knowledge.[138] As determined by the DAGs, the minimally sufficient adjustment set to estimate the total effect of malaria in the first trimester on any of the assessed maternal and birth outcomes was maternal age, maternal education, socioeconomic status, malnutrition, season coincident with the first trimester, and insecticide-treated nets (ITNs).

Maternal age was measured in years at enrollment. Maternal education was recorded at enrollment as no formal schooling, primary education (1-6 years of schooling), secondary education (7-12 years of schooling), or university and beyond education (≥13 years of schooling). Socioeconomic status was developed using the Global Network Socioeconomic Status Index (detailed above as SES was a predictor for Aim 1).[136] As a proxy for malnutrition, we used maternal body-mass index (BMI) at enrollment. Maternal BMI was calculated from the maternal weight recorded at enrollment in kilograms divided by the maternal height recorded at enrollment in meters squared. Season coincident with the first trimester of pregnancy was defined as rainy or not-rainy based on the collection date recorded on the malaria DBS filter paper sample with the specific months defined as rainy varying across countries: April to October in DRC,[133] April to June and October to November In Kenya,[134] and November to April in Zambia.[135] As we were limited to data collected by the ASPIRIN trial, we did not have data on ITNs and did not adjust for ITN use in our causal model.

Based on previous studies of malaria infection in late pregnancy,[4,7,86] we examined the birth outcomes of preterm delivery, small for gestational age, low birth weight, and perinatal mortality and the maternal outcomes of anemia in late pregnancy and hypertensive disorders of pregnancy. All birth outcomes were assessed as the prevalence at birth. The maternal outcome of anemia in late pregnancy was assessed as prevalence at late pregnancy (26-30 weeks of gestation). The maternal outcome of hypertensive disorders was assessed as prevalence during the period from late pregnancy to delivery (20 weeks of gestation up until 42 days following delivery).

The main outcome of interest, preterm birth, was defined as a stillbirth or live birth at or after 20 weeks and 0 days of gestation and before 37 weeks and 0 days of gestation.[19] Birth attendants are trained to collect the data and assess outcomes as part of the Global Network Maternal Newborn Health Registry.[130]

Secondary birth outcomes of interest included small for gestational age, low birth weight, and perinatal mortality. Small for gestational age was defined as any live birth whose birth weight was measured within 4 days of delivery and the birth weight is below the INTERGROWTH 10th percentile for a given gestational age and sex of the newborn.[19] Low birth weight was defined as a measured birthweight of < 2500 g measured within 4 days of delivery.[19] Birthweight is the first weight of the fetus or newborn obtained after birth, ideally measured within the first hour of life.[19] Perinatal mortality was defined as mortality in the perinatal period beginning at 20 completed weeks of gestation (154 days of gestation) and ending at seven completed days after birth.[19] Perinatal mortality included stillbirths and deaths in the first week of life.[19] Miscarriages with a gestational age of 20 weeks or greater were classified as stillbirths and thus included in the perinatal mortality definition[19]

Secondary maternal outcomes of interest included anemia in late pregnancy and hypertensive disorders of pregnancy. Anemia in late pregnancy was defined as hemoglobin level <11 g/dL measured between 26-30 weeks of gestation, based on WHO cut-offs for pregnant women between 26-30 weeks of gestation.[139] Hypertensive disorders were defined as any of the following: (1) 2 consecutive time points with ≥140 mm Hg systolic or ≥90 mm Hg diastolic where those two timepoints occur more than 7 days apart. The criteria (i.e., elevated systolic or elevated diastolic) must be consistent

for the two consecutive visits, or (2) Have evidence of hypertensive disease such as: any reported severe adverse event of preeclampsia or eclampsia, Maternal and Newborn Health registry report of hypertensive disease, preeclampsia or eclampsia, or reports of elevated blood pressure that meet the criteria based on the American College of Obstetricians and Gynecologists 2013 “Hypertension in Pregnancy” Task Force Report at any point after 20 weeks of gestation AND have at least one report of elevated blood pressure that is not followed by a normal value, in which case their outcome classification was decided by masked clinical experts[19]

Data on all confounders included except season coincident with first trimester and all maternal and birth outcomes drew on the extant ASPIRIN study data which was reported by each of the sites to the Global Network Data Coordinating Center (DCC) (RTI International).[18] Data quality was ensured as described above for Aim 1.[18]

Additional variables included projected gestational age at enrollment, antenatal care visits, delivery attendant, delivery location, and delivery mode. Projected gestational age at enrolment as described above in Aim 1 was developed from an algorithm described in Hoffman et al., 2020, and further details are presented in the Supplementary Information for Chapter IV on page 119. Antenatal care visits were recorded by the MNH registry and were the number of antenatal care visits recorded on the perinatal form. Delivery attendant was also recorded by the MNH registry and was categorized into four groups: (1) physician, (2) nurse, nurse midwife, lady health worker or health worker, (3) traditional birth attendant, or (4) family, self or other. Delivery location was recorded by MNH registry as one of three options: hospital, clinic or health center, or home or other. Delivery mode was recorded by the MNH registry as one of

four groups: vaginal (with or without forceps/vacuum), C-section, miscarriage, or medical termination of birth. As Aim 2 restricted to women who provided any post-baseline outcome data and have delivered at 20 weeks of gestational age or greater, there were no outcomes of miscarriages or medical termination of birth within our analysis population.

Aim 2 Analysis

The main exposure of interest was malaria in the first trimester. Based on previous studies of malaria infection in late pregnancy,[4,7,86] we examined the birth outcomes of preterm delivery, small for gestational age, low birth weight, and perinatal mortality, and the maternal outcomes of anemia in late pregnancy and hypertensive disorders during pregnancy. All birth outcomes were assessed as the prevalence of the outcome at birth.[140]

The analysis population included all randomized participants who provided any post-baseline outcome data and who delivered at 20 weeks of gestational age or greater. This restriction on the data analysis was performed in recognition that missing data were likely to occur because of miscarriage or medical termination of pregnancy.[19] Because of this restriction on the data analysis, we were determining prevalences of maternal and birth outcomes.

Crude prevalence ratios (PRs) and prevalence differences (PDs) were calculated for each maternal and birth outcome by malaria in the first trimester, using stratified 2x2 tables for each country. We present 99% confidence intervals to account for multiple comparisons. We did not calculate the crude prevalence ratio if there were no cases of the outcome when stratified by malaria in the first trimester exposure and country.

To assess whether the crude results from each country were too heterogeneous to combine, we calculated the I^2 value. If I^2 exceeded a pre-specified threshold of 40%,[137] we did not pool results across countries to calculate a summary estimate. If the I^2 value was ≤40%, we used the DerSimonian and Laird inverse variance method to calculate a summary estimate.[137] We did not pool results of any outcome that had zero participants in at least one of the cells when stratified by malaria in the first trimester exposure and country.

After using DAGs to identify potential confounders, we determined that the minimally sufficient adjustment set to estimate the total effect of malaria in the first trimester on any of the assessed maternal and birth outcomes was maternal age, maternal education, socioeconomic status, malnutrition, season coincident with the first trimester, and insecticide-treated nets (ITNs). As a proxy for malnutrition, we used maternal body-mass index (BMI) at enrollment.[141]

To model the continuous covariates of maternal age, BMI, and SES, we used restricted cubic splines with four knots at the 5th, 35th, 65th, and 95th percentiles to maximized flexibility of our models. For Kenya SES, the 35th and 65th percentiles were identical and so we used restricted cubic splines with three knots at the 5th, 50th, and 95th percentiles. Maternal education was categorized into two categories: lower (no more than primary education (1-6 years of schooling)), and higher (at least some secondary education (7-12 years of schooling) or university and beyond education (≥13 years of schooling)). Season coincident with the first trimester of pregnancy was either rainy or non-rainy. As we were limited to data collected by the ASPIRIN trial, we did not have data on ITNs and did not adjust for ITN use in our causal model.

Our main analytic approach was parametric g-computation.[142] We estimated the average causal effect of malaria in the first trimester on the assessed maternal or birth outcome. We used parametric g-computation to determine the predicted outcomes given all participants had the exposure of malaria in the first trimester and the predicted outcomes given all participants did not have the exposure of malaria in the first trimester.

We initially attempted to use linear binomial models to estimated adjusted prevalence difference and log-binomial to estimated adjusted prevalence ratios. However, our models did not converge, so we instead used logistic regression as our model as logistic regression always converges and provides a probability within 0 and 1.

We first modeled the association of malaria in the first trimester and the assessed maternal or birth outcome. We used logistic regression and adjusting for confounders identified by the DAG. We estimated the parameter coefficients of the model, and then predicted the mean outcome given everyone was exposed and given everyone was unexposed. The mean outcome is the weighted average of the mean outcomes for the combination of values for the confounders included, i.e., the standardized outcome.

Our logistic regression model provided the predicted outcomes as the predicted log-odds. We converted these predicted log-odds to estimated mean prevalences by first exponentiating the predicted log odds to obtain predicted odds, and then as prevalence is equal to odds divided by the denominator of odds plus 1, calculating the predicted prevalences from the predicted odds. We then took the mean of the predicted prevalences to estimate the mean prevalence of the outcome given everyone was

exposed to malaria in the first trimester and given everyone was unexposed. From these mean prevalence estimates, we calculated the adjusted prevalence ratios (aPRs) and adjusted prevalence differences (aPDs). We then used bootstrapping with 10,000 repetitions to determine their corresponding 99 percentile confidence intervals by selecting the 50th ordered value as the lower limit and 9950th ordered value as the upper limit.

We thus estimated the adjusted association of malaria in the first trimester on the assessed outcome. However, our goal was to estimate the causal effect of malaria in the first trimester on adverse maternal and birth outcomes. In order to interpret our results of our g-computation approach as an estimate of the average causal effect, we would need to assume counterfactual consistency, exchangeability, and positivity.[142] Counterfactual consistency requires that the observed outcomes among those who were exposed (or not) is equal to the potential outcomes that would be observed given they were exposed (or not).[142] Exchangeability exists when the potential outcomes under the different exposures are independent of the observed exposures.[142] This assumption could be met by conditional mean exchangeability (i.e., the potential outcomes were independent of the observed exposure given the covariates). Conditional mean exchangeability assumes that all confounding has been controlled in the model.[142] The final assumption of positivity requires a non-zero probability for all values of exposure (i.e., malaria in the first trimester or not) conditional on the variables required for conditional mean exchangeability.[142] These assumptions of counterfactual consistency, conditional mean exchangeability, and positivity must be met before we could interpret the adjusted association of malaria in the first trimester on the outcome

as an estimate of the average causal effect.

In our model, trial arm was randomly allocated without respect to malaria in the first trimester status and thus we did not need to control for the effects of the ASPIRIN study protocol. We did not adjust for malaria in late pregnancy as it is on the causal pathway of malaria in the first trimester and any of the assessed outcomes. We also did not assess the adjusted effect of malaria in the first trimester on hypertensive disorders as there was not a nonzero probability of malaria infection for all values of the confounders that would be included in the model. As we were limited to data collected by the ASPIRIN trial, we did not have data on ITNs and did not adjust for ITN use in our causal model.

CHAPTER IV: PREDICTORS OF PLASMODIUM FALCIPARUM INFECTION IN THE FIRST TRIMESTER AMONG NULLIPAROUS WOMEN FROM THE DEMOCRATIC REPUBLIC OF THE CONGO, KENYA, AND ZAMBIA

Introduction

Malaria is a serious global health issue, with an estimated 228 million cases annually and 411,000 associated deaths worldwide in 2018.[24] Nearly 85% of malaria deaths globally occurred in 21 countries, mostly in children under-5 in sub-Saharan Africa.[2] In addition to children, pregnant women constitute a high-risk group, and in sub-Saharan Africa, 29% of all pregnancies are exposed to malaria infection.[2] Malaria infection in pregnancy can cause maternal anemia, preterm birth, stillbirth, and low birth weight.[1] Despite the known perinatal complications of malaria infection in pregnancy, our understanding of predictors associated with malaria infection in pregnancy is limited to studies that evaluated malaria after 20 weeks of gestation. These studies show that factors associated with malaria infection in pregnancy include low gravidity, rainy transmission season, young age, short stature, HIV infection, maternal anemia, earlier trimester of pregnancy, lower body-mass index (BMI), lower education, and low socioeconomic status.[6,7,17,31,43,57,94,95,97–103,105,106] However, data is limited about the predictors for malaria in the first trimester, rendering incomplete our understanding of the epidemiology of first-trimester *Plasmodium falciparum* infections.

The first trimester represents a potential target for intervention to prevent the negative consequences of malaria in pregnancy. Malaria in the first trimester infection

could impact the placentation process by inhibiting trophoblast invasion and disrupting placentation, and thus affect fetal growth.[9,10,55,68,78] Adverse changes in placentation could be even more pronounced among primigravidae (women pregnant for the first time) or nulliparous women (women who have never given birth) and among women living in high malaria transmission areas.[11] Women in their first pregnancy are particularly vulnerable to malaria, as women naturally acquire resistance to malaria in successive pregnancies by developing antibodies that inhibit binding of *P. falciparum*-infected erythrocytes to the placenta in subsequent pregnancies.[12,13] Thus, women who lack this parity-dependent immunity that reduces placental and peripheral parasitemia are unable to clear placental parasites quickly, leading to higher placental and peripheral parasitemia, and chronic placental infection, which is associated with low birth weight and maternal anemia.[13–17]

Despite the deleterious effects of malaria in early pregnancy when placentation is occurring, current treatment and prevention strategies habitually leave women unprotected from malaria. To treat and prevent incident malaria infections in pregnancy, the World Health Organization recommends prompt diagnosis and treatment of malaria, intermittent preventive therapy in pregnancy with sulfadoxine-pyrimethamine (IPT-SP), and use of insecticide-treated nets (ITNs).[2] However, in many malaria-endemic regions, diagnosing malaria in pregnancy is difficult because pregnant women frequently have parasite densities below the detection limit of common diagnostic tools. IPT-SP is not initiated until the second trimester because antenatal care (ANC) typically begins around 20 weeks of pregnancy, and IPT-SP is not recommended earlier in pregnancy because of potential teratogenic effects. Most pregnant women in malaria-endemic

settings, especially those in their first pregnancy, do not use ITNs because of discomfort, lack of ownership, and difficulties hanging the net.[3–8] These difficulties to diagnosing, treating, and preventing malaria leave women under-protected from malaria in the first trimester.

Many women in malaria endemic areas do not receive antenatal care (ANC) until the second trimester, which limits efficient observation of malaria in the first trimester.[77] Only two studies to have assessed predictors of malaria in early pregnancy.[22,88] These studies did not find a relationship between malaria in early pregnancy and maternal age, maternal height, maternal body mass index, socioeconomic status, or season that coincided with early pregnancy and found that women with lower overall educational attainment (no secondary education) were more likely to have malaria in early pregnancy.[22,88] However, one study used wide intervals to define early pregnancy (i.e., less than 120 days) instead of using the first trimester, and both included mostly multigravidae and were all single-site studies.[22,88] No study has assessed malaria in the first trimester across multiple transmission settings.

In this work, we leverage a large clinical trial to efficiently develop the first multi-country study of malaria in the first trimester among nulliparous women. Our study objective was to determine the prevalence of malaria in the first trimester across multiple transmission settings and characterize the predictors that are associated with increased likelihood of malaria in the first trimester. Based on previous work, we hypothesized that lower overall educational attainment (no secondary education) would be associated with increased likelihood of malaria in the first trimester

Methods

Study Design and Sample

We conducted a sub study of the *Eunice Kennedy Shriver* NICHD Global Network for Women's and Children's Health Research's trial of low-dose aspirin for the prevention of preterm delivery in nulliparous women with a singleton pregnancy (the ASPIRIN Trial).[18,19] Briefly, the ASPIRIN trial was a prospective, randomized, multi-national clinical trial that tested the hypothesis that low-dose acetylsalicylic acid reduces the risk of preterm birth when administered in the first trimester.[19] The trial recruited 11,920 nulliparous women between March 23, 2016 and April 11, 2019 at seven research sites in six countries (Democratic Republic of the Congo, Zambia, Guatemala, Pakistan, Kenya, and two sites in India),[19] from primary health-care centers and hospital-based clinics, and were initially screened for potential eligibility, specifically if they were nulliparous and pregnant between 6 weeks 0 days and 13 weeks 6 days based on last menstrual period.[18] Following enrollment, ultrasound was used to determine gestational age.[18]

The inclusion criteria from the ASPIRIN trial included nulliparous women between 18-40 years of age (possibility of minors who were 14 or older being enrolled if allowed by the country's guidelines) who were residents of the study area, had no more than two previous first-trimester pregnancy losses, and no medical contraindications to acetylsalicylic acid.[19] In addition, all women must have had a single live intrauterine pregnancy that was between 6 weeks and 0 days to 13 weeks and 6 days in gestational age that was confirmed by an early dating ultrasound.[19]

Women were excluded if they were already taking daily acetylsalicylic acid for more than a week or had a multiple gestation pregnancy.[19] Women were also excluded if a fetal anomaly was detected by ultrasound at screening, severe maternal anemia was present at screening (hemoglobin < 7.0 g/dL), systolic blood pressure ≥ 140 mm Hg or diastolic ≥ 90 mm Hg was present at screening, or if women had any medical condition that could be a contradiction to receiving acetylsalicylic acid (e.g., Type 1 diabetes, lupus, hypertension, or other significant disease), as evaluated by the site investigator.[19]

The sub-Saharan African study sites were located in Nord-Ubangi and Sud-Ubangi provinces in the Democratic Republic of the Congo; Bungoma, Busia, and Kakamega provinces in Kenya, and Kafue and Chongwe districts in Zambia (**Figure 4.1**). Each site had multiple recruitment locations.

Participant Data and Sample Collection and Processing

At enrollment, information was collected on demographics, including years of maternal age and education, pregnancy and medical history, and current medical information, including height in centimeters, weight in kilograms, blood pressure, heart rate, and diabetes.[19] Dried blood spots (DBS) were also collected by pricking the participant's finger and placing three blood spots on filter paper, which were then completely dried before storage in plastic bags with desiccant. Eligible women who consented for the sub-study were enrolled from January 2016 to April 2018.

DBS were shipped to the Meshnick Lab at the University of North Carolina at Chapel Hill in Chapel Hill, North Carolina where each was tested in duplicate for *P. falciparum* lactate dehydrogenase (pfldh) DNA using quantitative polymerase chain

reaction (qPCR), a sensitive detection method.[131] A *P. falciparum*-positive sample was defined as a sample when florescence for both replicates crossed the threshold prior to the 39th cycle, or when one replicate did not amplify and the other crossed the threshold prior to the 39th cycle. Discordant results between duplicates were excluded from analysis.

Exposure and Outcome Assessment

Based on previous studies of malaria infection in late pregnancy, we examined the following predictors: maternal age, maternal height, maternal body-mass index (BMI), maternal education, season that coincided with the first trimester of pregnancy, and socioeconomic status (SES). We used the Global Network Socioeconomic Status Index to assess socioeconomic status.[136] Developed from around 50,000 households of pregnant women included in the Global Network, the Socioeconomic Status Index was calculated by determining the sum score of 10 specific items (finished floor material, flush toilet, improved source of drinking water, electricity, television, smartphone, car, motorbike, use of liquefied petroleum gas or electricity for cooking fuel, or refrigerator) owned within the household. The scores were converted to a country-specific SES score that ranged from 0 to 100 with higher scores indicating better housing conditions and more household assets.[136] For predictors of maternal age, maternal height, maternal BMI, and socioeconomic status that were measured as continuous variables , we created two categories by dividing at the country-specific median based on data from this sub-study. Maternal education was categorized into two categories: lower (i.e., no formal schooling and primary education (1-6 years of schooling)), and higher (i.e.,

secondary education (7-12 years of schooling) and university and beyond education (≥13 years of schooling).

The season that coincided with the first trimester of pregnancy was defined as rainy or not-rainy, with the specific months defined as rainy varying across countries: April to October in DRC,[133] April to June and October to November In Kenya,[134] and November to April in Zambia.[135]

The main outcome of malaria in the first trimester was defined as *P. falciparum* positive status through qPCR in a sample obtained in the first trimester.

Statistical Analysis

Crude prevalence ratios (PRs) and prevalence differences (PDs) were calculated for malaria in the first trimester by each variable, using stratified 2x2 tables for each country. We present 99% confidence intervals to account for multiple comparisons and limit inflation of non-coverage rates.

To assess heterogeneity by country, we calculated the I^2 value. If I^2 exceeded a pre-specified threshold of 40%,[137] we did not pool results across countries to calculate a summary estimate. If the I^2 value was ≤40%, we used the DerSimonian and Laird inverse variance method to calculate the summary estimate.[137]

Comparisons were limited to observations without missing data for each variable. All analyses were conducted using the R statistical platform (version 4.0.2).

Ethical Considerations

The ASPIRIN trial protocol was approved by all the sites' and partner institutions' ethics review committees.[18] Research personnel obtained informed consent from all participants.[18] The current analysis was added to the ASPIRIN trial protocol Institutional

Review Board application and received ethical approval from The University of North Carolina at Chapel Hill Office of Human Research Ethics, Chapel Hill, NC, USA as well as approval by each of the participating sites and the data coordinating center, RTI International.

Results

The ASPIRIN trial enrolled 11,953 nulliparous pregnant women in the first trimester; 3,800 of these were enrolled from sub-Saharan sites: 1,362 from DRC, 1,400 from Kenya, and 1038 from Zambia. For the malaria sub-study, we analyzed a convenience sample of 1513 women (485 from DRC, 677 from Kenya, and 351 from Zambia) (**Figure 4.2**). Among these women, there were no missing data except for socioeconomic status (missing n=53).

Most of the nulliparous women included in this study were under 20 years of age, recruited before 12 weeks of gestation, and had an education level of secondary or university and beyond (**Table** 4.1). Compared to Kenyan or Zambian women, Congolese women were shorter, had lower BMI, and had a lower overall educational attainment (no secondary education).

Population characteristics and prevalence of malaria in the first trimester

The overall prevalence of first-trimester *P. falciparum* by qPCR was 38.5% (583/1513), though this varied by site: 62.9% (305/485 [95% CI: 58.6, 67.2]) in the DRC; 37.8% (256/677 [95% CI: 34.2, 41.5]) in Kenya; and 6.3% (22/351 [95% CI: 3.7, 8.8]) in Zambia (**Figure 4.1**).

Across the entire study population, compared to uninfected women, infected women were slightly younger (18 years vs. 19 years) and had lower overall educational

attainment (60% vs. 82% with higher education level). In addition, women with malaria in the first trimester were shorter in stature (155 cm vs. 157 cm), had lower weight (52 kg vs. 54 kg), and had slightly lower BMI (21.6 kg vs. 21.9 kg) (data not shown).

Factors correlated with malaria in the first trimester

In Kenya, women younger than 20 years old were more likely to have malaria in the first trimester compared to women 20 years or older (PR = 1.57 [99% CI: 1.21, 2.03]), with a corresponding prevalence difference of 0.17 [99% CI: 0.07, 0.26]. Also, Kenyan women who were 157 cm or shorter were more likely to have malaria in the first trimester than women taller than 157 cm [PR = 1.35 [99% CI: 1.04, 1.75]; PD = 0.11 [99% CI: 0.02, 0.21]). There were no relationships between maternal BMI, maternal education, or season that coincided with the first trimester, or socioeconomic status and prevalence of malaria in the first trimester in Kenyan women. Similarly, in the DRC and Zambia, there were no statistically-significant associations between predictors examined and prevalence of malaria in the first trimester (**Table 4.2**)

Pooling results

For maternal education, the results of the heterogeneity assessment supported pooling across countries (for PD: $I^2 = 0$ [99% CI: 0, 93]; for PR: $I^2 = 33$ [99% CI: 0, 97]) (**Figure 4.3**). Across all three study countries, the summary prevalence difference and 99% confidence interval indicated that lower vs. higher educational attainment was associated with higher prevalence of malaria in the first trimester (summary PD = 0.09 [99% CI: 0.01, 0.17]). Although not statistically significant, we observed a consistent elevated summary prevalence ratio and 99% confidence interval (summary PR = 1.28 [99% CI: 0.97, 1.70]).

For season during the first trimester, the results of the heterogeneity assessment again supported pooling (for PD: I^2 = 27 [0, 96]; for PR: I^2 = 0 [99% CI: 0, 94]). Across all three study countries, the summary prevalence difference and summary prevalence ratio suggested no change in prevalence of malaria in the first trimester based on the lack of statistical significance (summary PD = 0.00 [99% CI: -0.06, 0.06]; summary PR = 1.06 [99% CI: 0.91, 1.23]).

Discussion

We are the first study to report parasite prevalence in the first trimester among a large number of women from sites within these three countries representing multiple transmission settings. The malaria in the first trimester prevalence among 485 Congolese women was 62.9%, among 677 Kenyan women was 37.8%, and among 351 Zambian women was 6.3%. We determined that lower overall educational attainment was associated with higher prevalence of malaria in the first trimester in pooled estimates developed from women in all three countries, and younger age and shorter height were associated with higher prevalence of malaria in the first trimester among Kenyan women. The general lack of association between parasite prevalence in the first trimester in primigravidae and traditional predictors suggests that routine screening may be necessary to mitigate the potential effects of these infections for neonates.

When stratified by country, we found no significant association between maternal age or maternal height and malaria in the first trimester among Congolese or Zambian women, but did find that younger or shorter Kenyan women had higher prevalence of malaria in the first trimester. Maternal height and age have been inconsistently reported as predictors for malaria in early pregnancy. For example, previous studies conducted

in Tanzania and Benin did not find an association between age or height (studied only among Tanzanian women) and malaria prevalence in early pregnancy.[22,88] These studies included 121 Tanzanian women, of whom 48 were primigravidae, and 387 Beninese women, of whom 30 were primigravidae.[22,88] Compared to these previous studies, we had a larger sample size in Kenya (n=677) and focused on primigravidae which may have allowed us to detect the relationship between younger age or shorter height and higher prevalence of malaria in the first trimester.

Across all three countries with different transmission intensities, we saw elevated prevalences of malaria in the first trimester among women with lower overall educational attainment (i.e. no secondary education). When we combined data from all three countries to develop pooled estimates, lower overall educational attainment was significantly associated with higher prevalence of malaria in the first trimester. These findings are consistent with other studies. Schmiegelow et al. had previously found this relationship, and we were able to confirm this association using the same education level comparison (i.e., primary education level or less vs. higher educational attainment) with a much larger sample (1513 compared to 121 women) and a more precise time period for malaria in the first trimester (cutoff of 14 weeks compared to 17 weeks of gestation).[88]

When stratified by country, we did not find a significant relationship between lower maternal BMI, lower socioeconomic status, or rainy season coincident with the first trimester and higher prevalence of malaria in the first trimester. Previous studies also did not find any significant relationship between these predictors and malaria in

early pregnancy [22,88] In contrast, these factors have been associated with higher prevalence of malaria in later pregnancy.[7,94,95,98,99,103]

Most studies examining the relationship between age, education, and malaria in later pregnancy report an association between younger age or lower overall educational attainment and higher prevalence of malaria in later pregnancy.[4,6,7,9–20] Consistent with findings from malaria in later pregnancy, we found that younger age was associated with higher prevalence of malaria in the first trimester among Kenyan women in a moderate malaria transmission setting, and lower overall educational attainment (no secondary education) was associated with higher malaria prevalence in pooled estimates developed from women in all three sites. In addition, short stature has been associated with higher prevalence of malaria in late pregnancy, and we found that shorter height was associated with higher malaria in the first trimester prevalence among Kenyan women.[98]

Determining the prevalence of malaria in the first trimester can be useful to identify and quantify the potential problem, and identification of predictors can direct preventative strategies to women at highest risk. We leveraged a multi-national clinical trial and used qPCR, a very sensitive form of detection of malaria infection during pregnancy, to determine parasite prevalence and predictors of malaria in the first trimester among a large number of women in three countries and across multiple transmission settings. We also predominantly studied women with the highest risk of malaria in pregnancy and consequent negative health outcomes, i.e., women who were primigravidae.[13] Over successive pregnancies, women naturally acquire resistance to malaria in pregnancy by developing antibodies that inhibit binding of *P. falciparum*-

infected erythrocytes to the placenta after a previous infection during pregnancy.[12,13] Thus, primigravid women lack this parity-dependent immunity that reduces placental and peripheral parasitemia.[13] While we studied nulliparous women, 94% of women (n=1420) in our study were primigravidae.

We were limited to data collected by the ASPIRIN trial that focused on low-dose aspirin, and thus did not have data to examine other malaria-specific predictors such as bed net use. Because pregnant women frequently have low parasite densities below the detection limit of common diagnostic tools, we used qPCR to detect malaria infection during pregnancy; however, the clinical significance of these "submicroscopic" infections is a matter of some debate.[3–6,45] The ASPIRIN trial recruited women starting at 6 weeks of gestation, and thus we did not have any data on women and their malaria in the first trimester prevalence prior to conception or during the earliest stages of pregnancy.[19] Finally, as our study population was restricted to nulliparous women, our results on predictors of malaria in the first trimester cannot be generalized to all pregnant women.

In conclusion, we found that in high-transmission settings, these first-trimester infections are very common, and some predictors were significant in Kenya and overall. Because of the differences of our findings on predictors across country, more research is needed on identifying actionable predictors. In the absence of consistent and practical predictors of malaria in the first trimester, routine screening for parasites could be considered as a tool to detect and mitigate these infections.

Table 4.1: Characteristics of the study participant population, stratified by country

Variable	DRC	KENYA	ZAMBIA
Included, N	485	677	351
Maternal age (years), N (%)			
< 20	410 (84.5)	316 (46.7)	204 (58.1)
20-29	68 (14.0)	356 (52.6)	144 (41.0)
> 29	7 (1.4)	5 (0.7)	3 (0.8)
Median (P25, P75)	18.0 (17.0, 18.0)	20.0 (18.0, 22.0)	19.0 (18.0, 21.0)
Projected gestation age at enrollment (weeks, days), N (%) [a]			
6, 0 - 7, 6	50 (10.3)	115 (17.0)	40 (11.4)
8, 0 - 9, 6	133 (27.4)	216 (31.9)	80 (22.8)
10, 0 - 10, 6	69 (14.2)	89 (13.1)	35 (10.0)
11, 0 - 11, 6	82 (16.9)	89 (13.1)	50 (14.2)
12, 0 - 13, 6	151 (31.1)	168 (24.8)	146 (41.6)
Median (P25, P75)	10.7 (9.0, 12.3)	10.0 (8.3, 11.9)	11.4 (9.1, 12.7)
Maternal education, N (%)			
Lower	310 (63.9)	43 (6.4)	44 (12.5)
Higher	175 (36.1)	634 (93.6)	307 (87.5)
Maternal height (cm)			
Mean (SD)	155.8 (6.6)	156.1 (8.9)	157.5 (6.4)
Median (P25, P75)	156.0 (151.0, 160.0)	157.0 (150.0, 162.5)	157.0 (154.0, 161.0)
Maternal BMI (kg/m^2)			
Mean (SD)	20.8 (2.2)	23.3 (3.5)	22.0 (3.3)
Median (P25, P75)	20.6 (19.2, 22.2)	22.8 (20.8, 25.0)	21.5 (20.0, 23.5)
Socioeconomic status [b]			
Mean (SD)	13.3 (9.6)	16.7 (17.1)	43.9 (25.7)
Median (P25, P75)	16.2 (6.2, 16.2)	8.4 (8.4, 24.6)	38.9 (25.6, 66.3)
Season coincident with the first trimester of pregnancy, N (%)			
Rainy	244 (50.3)	211 (31.2)	174 (49.6)
Non-rainy	241 (49.7)	466 (68.8)	177 (50.4)

[a] Projected gestational age at enrollment developed from algorithm described in Hoffman et al., 2020.
[b] Socioeconomic status developed using Global Network Socioeconomic Status Index described in Patel et al., 2020. SES was calculated by determining the sum score of the number of 10 specific items owned by the household that was converted to a country-specific SES score that ranged from 0 to 100.[136]

Table 4.2: Crude prevalence, crude prevalence ratio (PRs), crude prevalence differences (PDs) and 99% confidence intervals (CIs) for predictors of malaria in the first trimester for nulliparous women by malaria in the first trimester status in the Democratic Republic of Congo (DRC), Kenya, and Zambia

	DRC			KENYA			ZAMBIA		
	Prevalence (%, n/N)	PR (99% CI)	PD (99% CI)	Prevalence (%, n/N)	PR (99% CI)	PD (99% CI)	Prevalence (%, n/N)	PR (99% CI)	PD (99% CI)
Maternal age (years) [a]									
Younger	65.0 (139/214)	1.06 (0.89, 1.27)	0.04 (-0.08, 0.15)	46.8 (148/316)	1.57 (1.21, 2.03)	0.17 (0.07, 0.26)	8.0 (11/138)	1.54 (0.53, 4.46)	0.03 (-0.04, 0.10)
Older	61.3 (166/271)	REF	REF	29.9 (108/361)	REF	REF	5.2 (11/213)	REF	REF
Maternal height (cm) [b]									
Shorter	64.6 (177/274)	1.06 (0.89, 1.28)	0.04 (-0.07, 0.15)	43.4 (148/341)	1.35 (1.04, 1.75)	0.11 (0.02, 0.21)	6.7 (12/180)	1.14 (0.39, 3.32)	0.01 (-0.06, 0.07)
Taller	60.7 (128/211)	REF	REF	32.1 (108/336)	REF	REF	5.8 (10/171)	REF	REF
Maternal BMI (kg/m^2) [c]									
Lower	67.5 (166/246)	1.16 (0.97, 1.39)	0.09 (-0.02, 0.21)	33.7 (112/332)	0.81 (0.63, 1.05)	-0.08 (-0.18, 0.02)	8.0 (14/174)	1.78 (0.59, 5.39)	0.04 (-0.03, 0.10)
Higher	58.2 (139/239)	REF	REF	41.7 (144/345)	REF	REF	4.5 (8/177)	REF	REF
Maternal education									
Lower	66.1 (205/310)	1.16 (0.95, 1.41)	0.09 (-0.03, 0.21)	53.5 (23/43)	1.46 (0.99, 2.15)	0.17 (-0.03, 0.37)	11.4 (5/44)	2.05 (0.59, 7.11)	0.06 (-0.07, 0.19)
Higher	57.1 (100/175)	REF	REF	36.8 (233/634)	REF	REF	5.5 (17/307)	REF	REF
Season coincident with the first trimester of pregnancy									
Rainy	66.0 (161/244)	1.10 (0.92, 1.32)	0.06 (-0.05, 0.18)	37.4 (79/211)	0.99 (0.75, 1.30)	-0.01 (-0.11, 0.10)	5.2 (9/174)	0.70 (0.24, 2.08)	-0.02 (-0.09, 0.04)
Non-rainy	59.8 (144/241)	REF	REF	38.0 (177/466)	REF	REF	7.3 (13/177)	REF	REF
Socioeconomic status [d]									
Lower	62.4 (131/210)	0.97 (0.81, 1.16)	-0.02 (-0.14, 0.10)	41.8 (177/423)	1.28 (0.96, 1.71)	0.09 (-0.01, 0.19)	6.1 (11/181)	0.93 (0.32, 2.70)	0.00 (-0.07, 0.06)
Higher	64.4 (163/253)	REF	REF	32.6 (73/224)	REF	REF	6.5 (11/169)	REF	REF

[a] Median maternal age cut points (in years, younger vs. older): DRC: <18 vs. ≥18, Kenya: <20 vs. ≥20, Zambia: <19 vs. ≥19.
[b] Median maternal height cut points (in cm, shorter vs. taller): DRC: ≤156 vs. >156, Kenya: ≤157 vs. >157, Zambia: ≤157 vs. >157.
[c] Median maternal BMI cut points (in kg/m^2, lower vs. higher): DRC: ≤20.6 vs. >20.6, Kenya: ≤22.8 vs. >22.8, Zambia: ≤21.5 vs. >21.5.
[d] Median socioeconomic status cut points (lower vs. higher): DRC: <16.18 vs. ≥16.18, Kenya: ≤8.38 vs. >8.38, Zambia: ≤38.92 vs. > 38.92.

Figure 4.1: Map of malaria transmission intensity based on study site
The study locations are located in Nord-Ubangi and Sud-Ubangi provinces in the Democratic Republic of the Congo, in Bungoma, Busia, and Kakamega provinces in Kenya, and in Kafue and Chongwe districts in Lusaka province in Zambia. Malaria transmission is modeled from the *Plasmodium falciparum* parasite rate among children aged 2-10 (PfPR 2-10) in 2015 and ranges from 0.6 in Nord-Ubangi and Sud-Ubangi provinces in the Democratic Republic of the Congo to 0.0 in Lusaka province in Zambia. Data on the modeled PfPR 2-10 was obtained from the Malaria Atlas Project.[143]

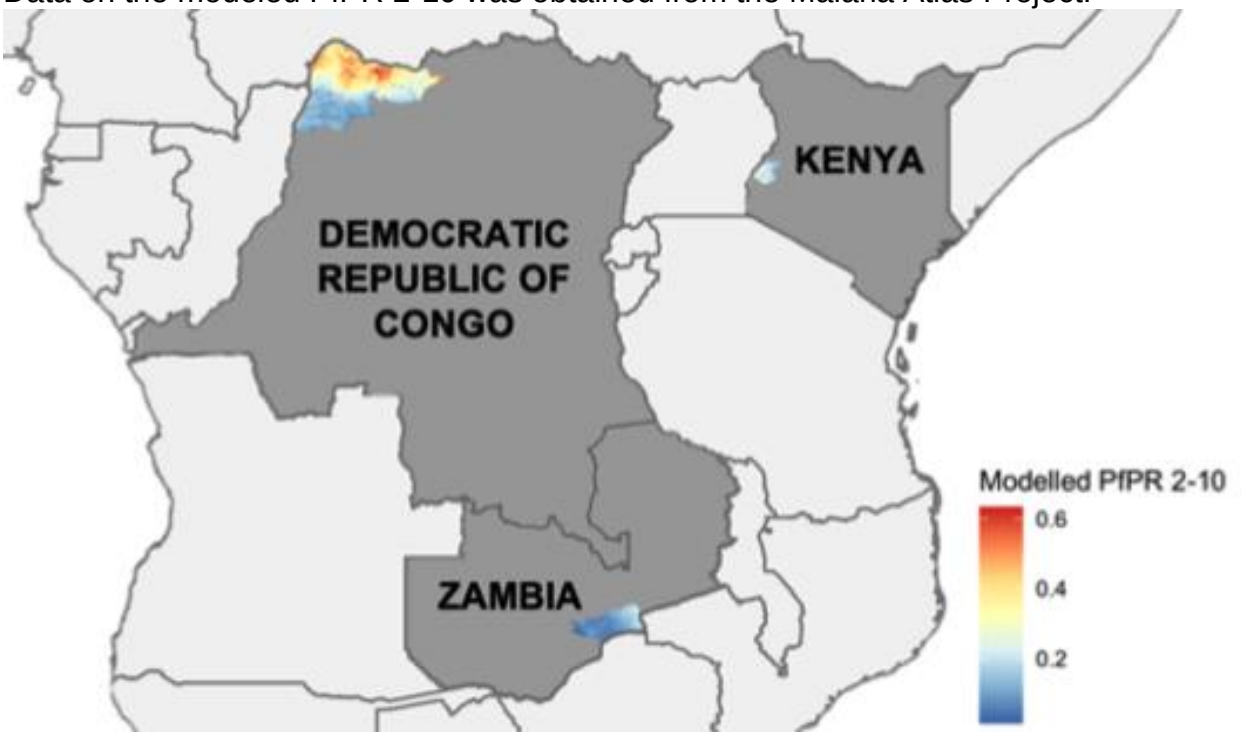


Figure 4.2: Study population of malaria predictors sub-study.
The ASPIRIN trial included 3800 women from Democratic Republic of the Congo (DRC), Kenya, and Zambia. From these women, we took a convenience sample of 1513 women for the malaria sub-study: 485 from DRC, 677 from Kenya, and 351 from Zambia.

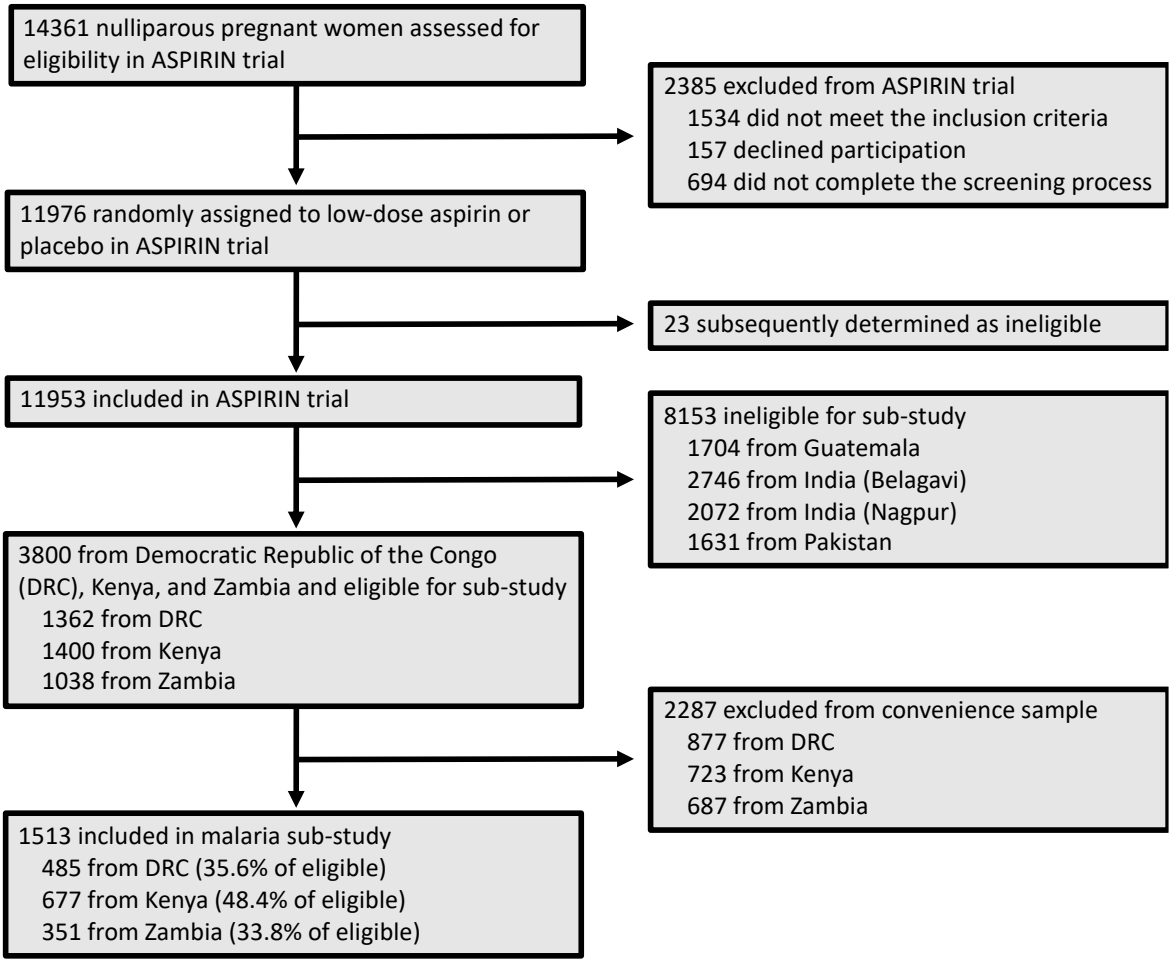
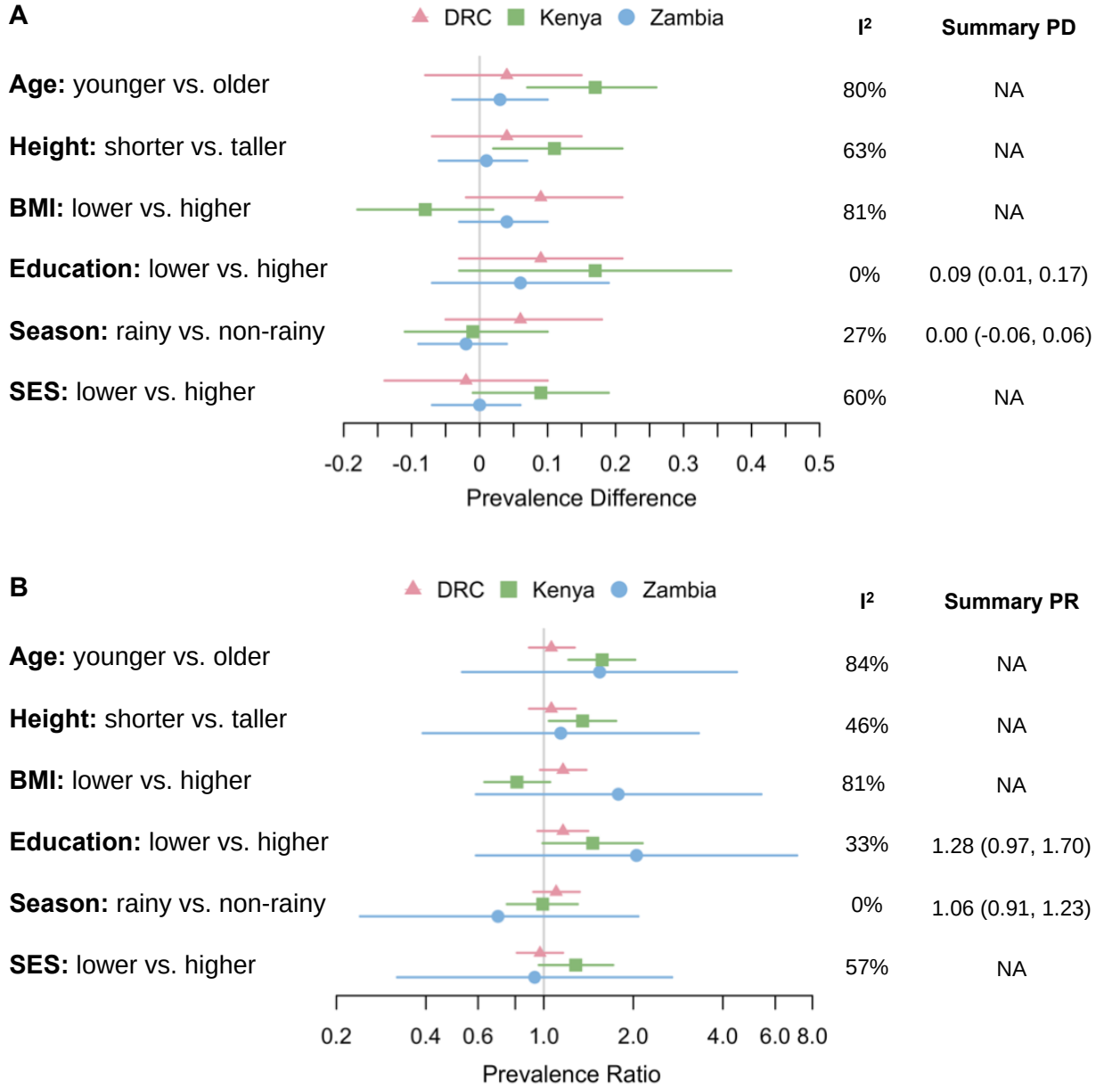


Figure 4.3: Comparison of predictors for malaria in the first trimester among nulliparous women from the Democratic Republic of Congo, Kenya, and Zambia
4.3A: Comparing using prevalence differences and 4.3B comparing using prevalence ratios. Pink triangles are DRC, green squares are Kenya, and blue circles are Zambia. Summary estimates were only presented if the I^2 value was less than 40%. The vertical grey line in the figure is the null value (0 for prevalence difference in 4.3A, 1 for prevalence ratio in 4.3B).



CHAPTER V: EFFECTS ON MATERNAL AND BIRTH OUTCOMES OF *PLASMODIUM FALCIPARUM* INFECTION IN THE FIRST TRIMESTER AMONG NULLIPAROUS WOMEN FROM THE DEMOCRATIC REPUBLIC OF THE CONGO, KENYA, AND ZAMBIA

Introduction

Malaria is a serious global health issue, with an estimated 228 million cases annually and 411,000 associated deaths worldwide in 2018.[24] Nearly 85% of global malaria deaths occurred in 21 countries, including the Democratic Republic of the Congo (DRC), Kenya, and Zambia.[2] In sub-Saharan Africa, 29% of all pregnancies are exposed to malaria infection.[2] Malaria infection in pregnancy can cause maternal anemia, preterm birth, stillbirth, and low birth weight.[1]

To treat and prevent incident malaria infections in pregnancy, the World Health Organization recommends prompt diagnosis and treatment of malaria, the use of intermittent preventive therapy in pregnancy with sulfadoxine-pyrimethamine (IPT-SP), and the use of insecticide-treated nets (ITNs).[2] However, these strategies are challenging to initiate for women early in their pregnancy and habitually leave women unprotected from malaria in the first trimester. For example, prompt treatment is delayed due to difficulties in diagnosing malaria in early pregnancy because pregnant women frequently have parasite densities below the detection limit of commonly available diagnostic tools. IPT-SP is not initiated until the second trimester because antenatal care (ANC) typically begins around 20 weeks of pregnancy, and IPT-SP is

contraindicated in the first trimester of pregnancy because of potential teratogenic effects. Many pregnant women in malaria-endemic settings, especially those in their first pregnancy in the first trimester, do not use ITNs because of discomfort associated with sleeping under the ITN, difficulties in hanging the net, and lack of ownership of ITNs.[3–8]

Despite the difficulties in treatment and prevention of malaria, the first trimester might represent a critical time for intervention to prevent the negative consequences of malaria in pregnancy. Malaria in the first trimester could impact placental development and thus affect fetal growth.[55,68,78] During the first trimester, the trophoblast invades and remodels the maternal uterine arteries in order to increase uterine artery blood flow.[48] Trophoblast invasion is essential for normal placental function and fetal growth, and occurs from very early in pregnancy until 18-20 weeks of gestation.[10] However, placentation is particularly sensitive to pathology in the first trimester, and malaria in early pregnancy could inhibit trophoblast invasion and cause disruptions in placentation.[9,10] In a study of 68 mostly multigravidae women, malaria in the first trimester was associated with a negative impact on placental vascular development and consequent pregnancy outcomes.[11,12]

Changes in placental vascularity would be even more pronounced among primigravidae or nulliparous women, and among women living in high malaria transmission areas.[11] Women in their first pregnancy are particularly vulnerable to malaria, as women naturally acquire resistance to malaria in pregnancy in successive pregnancies through the development of antibodies that inhibit binding of *Plasmodium falciparum*-infected erythrocytes to the placenta.[12,13] Thus, primigravid and nulliparous women, who lack this parity-dependent immunity reducing placental and peripheral

parasitemia are unable to clear placental parasites quickly leading to higher placental and peripheral parasitemia, and chronic placental infection, which is associated with low birth weight and maternal anemia.[13–17]

Research about effects of malaria in the first trimester is limited because most studies are limited to observations in the second trimester when women begin receiving antenatal care (ANC).[77] Most research has focused on the birth outcomes of low birth weight or the maternal outcome of anemia in late pregnancy or at delivery and has found conflicting results for either birth outcome and the association of malaria in early pregnancy.[4,6,11,20,39,84–86,88,92] In addition, three previous studies found conflicting results for the association of malaria in early pregnancy with preterm birth.[4,7,92] Most prior studies used wide gestational age intervals to define early pregnancy instead of limiting to the first trimester, included mostly multigravidae women, were all single-site studies.

In this work, we leverage an existing, large clinical trial to develop the first multi-country study of malaria in the first trimester among nulliparous women. Our study objective was to estimate the causal effect malaria in the first trimester on five maternal and birth outcomes: preterm delivery, small for gestational age, low birth weight, perinatal mortality, and anemia in late pregnancy. Based on previous work, we hypothesized that malaria in the first trimester would increase the prevalence of all the adverse maternal and birth outcomes assessed.

Methods

Rationale

We previously reported the malaria in the first trimester prevalence in three sites in sub-Saharan Africa. This analysis population was further restricted to all randomized

participants who provided any post-baseline outcome data and who delivered at 20 weeks of gestational age or greater. Thus, the malaria in the first trimester prevalence when restricted to this analysis population was 63.3% (297/469) in the DRC, 38.0% (244/642) in Kenya, and 6.3% (21/335) in Zambia. Since we had a large sample size of women from three sites with varying malaria prevalence, we had a unique chance to estimate the impact of malaria in the first trimester on five maternal and birth outcomes.

Study Design and Sample

We conducted a sub-study of the *Eunice Kennedy Shriver* NICHD Global Network for Womens' and Children's Health Research (GN)'s trial of low-dose aspirin for the prevention of preterm delivery in nulliparous women with a singleton pregnancy (the ASPIRIN Trial).[18,19] Briefly, the ASPIRIN trial was a prospective, randomized, multi-national clinical trial that tested the hypothesis that low-dose acetylsalicylic acid initiated in the first trimester reduces the risk of preterm birth.[19] The trial recruited 11,920 nulliparous women at seven research sites in six countries (DRC, Kenya, Zambia, Guatemala, Pakistan, and India) between March 23, 2016 and April 11, 2019, who were randomly assigned 1:1, beginning in the first trimester (gestational age between 6 weeks, 0 days and 13 weeks, 6 days, confirmed by study ultrasound) and continuing until 36 weeks and 7 days of gestation or delivery, to receive either daily low-dose acetylsalicylic acid (81 mg dose) or a visually identical placebo.[19]

Women were recruited by community health workers from primary health-care centers and hospital-based clinics, and were screened for eligibility. Women were asked if they were nulliparous and pregnant between 6 weeks 0 days and 13 weeks 6 days

based on last menstrual period.[18] Ultrasound was used to determine gestational age after enrollment.[18]

Nulliparous women were included if they were between 18-40 years of age (some minors were included if country-specific guidelines permitted), were residents of the study area, and had no more than two previous first-trimester pregnancy losses. In addition, all women must have had a single live intrauterine pregnancy that was between 6 weeks and 0 days to 13 weeks and 6 days in gestational age, confirmed by an early dating ultrasound.

Women were excluded if they were already taking daily acetylsalicylic acid for more than a week, if they were pregnant with multiple gestations, and if they had severe anemia at screening (hemoglobin < 7.0 g/dL), systolic blood pressure ≥ 140 mm Hg or diastolic ≥ 90 mm Hg at screening, or any medical condition that could be a contradiction to receiving acetylsalicylic acid (e.g., Type 1 diabetes, lupus, hypertension, or other significant disease), as evaluated by the site investigator.[19] Further exclusion criteria included a fetal anomaly detected by ultrasound at screening.[19] In addition, to be included in this analysis population assessing maternal and birth outcomes, participants had to provide any post-baseline outcome data and have delivered at 20 weeks of gestational age or greater.

The sub-Saharan African study sites were located in Nord-Ubangi and Sud-Ubangi provinces in the DRC; Bungoma, Busia, and Kakamega provinces in Kenya, and Kafue and Chongwe districts in Zambia (**Figure** 5.1). Each site had multiple recruitment locations.

Participant Data and Sample Collection and Processing

At enrollment, information was collected on demographics (including years of maternal age and education), pregnancy and medical history, and current medical information (including height in centimeters, weight in kilograms, blood pressure, heart rate, and history of diabetes).[19] Dried blood spots (DBS) were also collected by pricking the participant's finger and placing three blood spots on filter paper, which were then completely dried before storage in plastic bags with desiccant. We included all eligible women enrolled between January 2016 to April 2018 who consented to this sub-study.

DBS were shipped to the University of North Carolina in Chapel Hill, North Carolina, where each was retrospectively tested in duplicate for *P. falciparum* lactate dehydrogenase (pfldh) DNA using quantitative polymerase chain reaction (qPCR), a sensitive detection method.[131] DBS were processed in chronological arrival order and a convenience sample was included in this analysis. A *P. falciparum*-positive sample was defined as a sample when florescence for both replicates crossed the threshold prior to the 39th cycle, or when one replicate did not amplify and the other crossed the threshold prior to the 39th cycle. Discordant results between duplicates were excluded from analysis.

Maternal and birth outcomes were obtained at delivery and up to 42 days following delivery using the Global Network Maternal and Newborn Health Registry.[19]

Exposure, Confounder, and Outcome Assessment

The main exposure of interest was malaria in the first trimester, defined as a positive result for *P. falciparum* through qPCR in a sample obtained at enrollment.

To estimate the causal effect of malaria in the first trimester on the assessed maternal or birth outcome, we identified confounders of the exposure-outcome relationship using causal directed acyclic graphs (DAGs) based on prior knowledge.[138] As determined by the DAGs, the minimally sufficient adjustment set to estimate the total effect of malaria in the first trimester on any of the assessed maternal and birth outcomes was maternal age, maternal education, socioeconomic status, malnutrition, season that coincided with the first trimester, and insecticide-treated nets (ITNs).

Maternal age was measured in years at enrollment. Maternal education was recorded at enrollment as no formal schooling, primary education (1-6 years of schooling), secondary education (7-12 years of schooling), or university and beyond education (≥13 years of schooling). SES was calculated using the GN Socioeconomic Status Index.[136] Developed from around 50,000 households of pregnant women included in GN, the Socioeconomic Status Index is calculated by determining the sum score of 10 specific items owned by the household and converting to country-specific SES score.[136] As a proxy for malnutrition, we used maternal body-mass index (BMI) at enrollment. Maternal BMI was calculated from the maternal weight recorded at enrollment in kilograms divided by the maternal height recorded at enrollment in meters squared. Season that coincided with the first trimester of pregnancy was defined as rainy or not-rainy based on the collection date recorded on the malaria DBS filter paper sample with the specific months defined as rainy varying across countries: April to October in DRC,[133] April to June and October to November In Kenya,[134] and November to April in Zambia.[135] As we were limited to data collected by the ASPIRIN trial, we did not have data on ITNs and did not adjust for ITN use in our causal model.

Based on previous studies of malaria infection in late pregnancy,[4,7,86] we examined the birth outcomes of preterm delivery, small for gestational age, low birth weight, and perinatal mortality and the maternal outcomes of anemia in late pregnancy and hypertensive disorders of pregnancy. All birth outcomes were assessed as the prevalence at birth. The maternal outcome of anemia in late pregnancy was assessed as prevalence at late pregnancy (26-30 weeks of gestation). The maternal outcome of hypertensive disorders was assessed as prevalence during the period from late pregnancy to delivery (20 weeks of gestation up until 42 days following delivery).

The main outcome of interest, preterm birth, was defined as a stillbirth or live birth at or after 20 weeks and 0 days of gestation and before 37 weeks and 0 days of gestation.[19]

Secondary birth outcomes of interest included small for gestational age, low birth weight, and perinatal mortality. Small for gestational age was defined as any live birth whose birth weight was measured within 4 days of delivery and the birth weight is below the INTERGROWTH 10th percentile for a given gestational age and sex of the newborn.[19] Low birth weight was defined as a measured birthweight of < 2500 g measured within 4 days of delivery.[19] Birthweight is the first weight of the fetus or newborn obtained after birth, ideally measured within the first hour of life.[19] Perinatal mortality was defined as mortality in the perinatal period beginning at 20 completed weeks of gestation (154 days of gestation) and ending at seven completed days after birth.[19] Perinatal mortality included stillbirths and deaths in the first week of life. Miscarriages with a gestational age of 20 week of greater were classified as stillbirths and thus included in the perinatal mortality definition.[19]

The secondary maternal outcome of interest included anemia in late pregnancy, defined as hemoglobin level <11 g/dL measured between 26-30 weeks of gestation, based on World Health Organization cut-offs.[139] Another secondary maternal outcome of interest included was hypertensive disorders, defined as any of the following: (1) 2 consecutive time points with ≥140 mm Hg systolic or ≥90 mm Hg diastolic where those two timepoints occur more than 7 days apart. The criteria (i.e., elevated systolic or elevated diastolic) must be consistent for the two consecutive visits or (2) Have evidence of hypertensive disease such as: any reported severe adverse event of preeclampsia or eclampsia, Maternal and Newborn Health registry report of hypertensive disease, preeclampsia or eclampsia, or reports of elevated blood pressure that meet the criteria based on the American College of Obstetricians and Gynecologists 2013 “Hypertension in Pregnancy” Task Force Report at any point after 20 weeks of gestation AND have at least one report of elevated blood pressure that is not followed by a normal value, in which case their outcome classification was decided by masked clinical experts[19]

Statistical Analyses

The analysis population included all randomized participants who provided any post-baseline outcome data and who delivered at 20 weeks of gestational age or greater. This restriction on the data analysis was performed in recognition that missing data were likely to occur because of miscarriage or medical termination of pregnancy.[19] Because of this restriction on the data analysis, we determined prevalences of all birth outcomes, and the prevalence of anemia in late pregnancy and the prevalence of hypertensive disorders during pregnancy.[140]

Crude associations

Crude prevalence ratios (PRs) and prevalence differences (PDs) were calculated for each maternal and birth outcome by malaria in the first trimester, using stratified 2x2 tables for each country. We present 99% confidence intervals to account for multiple comparisons. We did not calculate the crude prevalence ratio if there were no cases of the outcome when stratified by malaria in the first trimester exposure and country.

To assess whether the results from each country were too heterogeneous to combine, we calculated the I^2 value. If I^2 exceeded a pre-specified threshold of 40%,[137] we did not pool results across countries to calculate a summary estimate. If the I^2 value was ≤40%, we used the DerSimonian and Laird inverse variance method to calculate a summary estimate.[137] We did not pool results of any outcome that had zero participants in at least one of the cells when stratified by malaria in the first trimester exposure and country.

Confounder identification and specification

After using DAGs to identify potential confounders, we determined that the minimally sufficient adjustment set to estimate the total effect of malaria in the first trimester on any of the assessed maternal and birth outcomes was maternal age, maternal education, socioeconomic status (SES), malnutrition, season that coincided with the first trimester of pregnancy, and insecticide-treated nets (ITNs). As a proxy for malnutrition, we used maternal body-mass index (BMI) at enrollment.[141]

To model the continuous covariates of maternal age, BMI, and SES, we used restricted cubic splines with four knots at the 5^{th}, 35^{th}, 65^{th}, and 95^{th} percentile to maximized flexibility of our models. For Kenya SES, the 35^{th} and 65^{th} percentiles were

identical and so we used restricted cubic splines with three knots at the 5th, 50th, and 95th percentiles. Maternal education was categorized into two categories: lower (no more than primary education (1-6 years of schooling)), and higher (at least some secondary education (7-12 years of schooling) or university and beyond education (≥13 years of schooling)). Season that coincided with the first trimester of pregnancy was either rainy or non-rainy. Because we were limited to data collected by the ASPIRIN trial, we did not have data on ITNs and did not adjust for ITN use in our causal model.

Adjusted effects

Our main analytic approach was parametric g-computation.[142] We estimated the average causal effect of malaria in the first trimester on the assessed maternal or birth outcome. We used parametric g-computation to determine the predicted outcomes given the all participants had the exposure of malaria in the first trimester and the predicted outcomes given all participants did not have the exposure of malaria in the first trimester.

We initially attempted to use linear binomial models to estimated adjusted prevalence difference and log-binomial to estimated adjusted prevalence ratios. However, our models did not converge, so we instead used logistic regression as our model as logistic regression always converges and provides a probability within 0 and 1.

We first modeled the association of malaria in the first trimester and the assessed maternal or birth outcome. We used logistic regression and adjusted for confounders identified by the DAG. We estimated the parameter coefficients of the model, and then predicted the mean outcome given everyone was exposed and given everyone was unexposed. The mean outcome is the weighted average of the mean

outcomes for the combination of values for the confounders included, i.e., the standardized outcome.

Our logistic regression model provided the predicted outcomes as the predicted log-odds. We converted these predicted log-odds to estimated mean prevalences by first exponentiating the predicted log odds to obtain predicted odds, and then as prevalence is equal to odds divided by the denominator of odds plus 1, calculating the predicted prevalences from the predicted odds. We then took the mean of the predicted prevalences to estimate the mean prevalence of the outcome given everyone was exposed to malaria in the first trimester and given everyone was unexposed. From these mean prevalence estimates, we calculated the adjusted prevalence ratios (aPRs) and adjusted prevalence differences (aPDs). We then used bootstrapping with 10,000 repetitions to determine their corresponding 99 percentile confidence intervals by selecting the 50^{th} ordered value as the lower limit and 9950^{th} ordered value as the upper limit.

We thus calculated the adjusted effect of malaria in the first trimester on the assessed outcome. However, our goal was to estimate the causal effect of malaria in the first trimester on adverse maternal and birth outcomes. In order to interpret our results of our g-computation approach as an estimate of the average causal effect, we would need to assume counterfactual consistency, exchangeability, and positivity.[142] Counterfactual consistency requires that the observed outcomes among those who were exposed (or not) is equal to the potential outcomes that would be observed given they were exposed (or not).[142] Exchangeability exists when the potential outcomes under the different exposures are independent of the observed exposures.[142] This

assumption could be met by conditional mean exchangeability (i.e., the potential outcomes were independent of the observed exposure given the covariates). Conditional mean exchangeability assumes that all confounding has been controlled in the model.[142] The final assumption of positivity requires a non-zero probability for all values of exposure (i.e., malaria in the first trimester or not) conditional on the variables required for conditional mean exchangeability.[142] These assumptions of counterfactual consistency, conditional mean exchangeability, and positivity must be met before we could interpret the adjusted effects of malaria in the first trimester on the assessed outcome as an estimate of the average causal effect.

In our model, trial arm was randomly allocated without respect to malaria in the first trimester status and was thus we did not need to control for the effects of the ASPIRIN study protocol. We did not adjust for malaria in late pregnancy as it is on the causal pathway of malaria in the first trimester and any of the assessed outcomes. As we were limited to data collected by the ASPIRIN trial, we did not have data on ITNs and did not adjust for ITN use in our causal model.

Comparisons were limited to observations without missing data for each variable, and models were limited to observations without missing data for all covariates and the outcome. All analyses were conducted using the R statistical platform (version 4.0.2).[126]

Ethical Considerations

The ASPIRIN trial protocol and malaria sub-study were approved by all the sites' and partner institutions' ethics review committees.[18] Research personnel obtained informed, written consent from all participants.[18]

Results

Population characteristics and prevalence of malaria in the first trimester

The ASPIRIN trial enrolled 11,953 nulliparous pregnant women in the first trimester; 3,800 of these were enrolled from sub-Saharan sites: 1,362 from DRC, 1,400 from Kenya, and 1,038 from Zambia. For the malaria analysis sub-study, we analyzed a convenience sample of 1,446 women (469 from DRC, 642 from Kenya, and 335 from Zambia) (**Figure** 5.2).

There were no missing data for preterm birth, or perinatal mortality. There were 92 (6.4% of total sample) women missing data for small for gestational age, 49 (3.4% of total sample) missing data for low birth weight, and 195 (13.5%) missing data for anemia in late pregnancy. There were no missing covariate data except for 40 (2.8% of total sample) women missing socioeconomic status.

Most of the nulliparous women included in this study were under 20 years of age, recruited before 12 weeks of gestation, and had at least some secondary education (**Table** 5.1). Congolese women were younger, shorter, had lower BMI, and had lower educational attainment compared to Kenyan or Zambian women. Most women had a nurse or midwife as a delivery attendant, delivered at a clinic or health center, and had a vaginal delivery.

Prevalence of adverse maternal and birth outcomes

The prevalence of preterm birth was highest in DRC (19.6%), lower in Kenya (9.0%), and lowest in Zambia (6.9%). Similarly, the prevalence of anemia in late pregnancy was highest in DRC (56.8%), lower in Kenya (35.5%), and lowest in Zambia (11.8%). The prevalence of perinatal mortality was similar in all three countries: 6.8%

(32/469) in DRC, 5.3% (34/642) in Kenya, and 5.4% (18/335) Zambia. Small for gestational age prevalence was high for both DRC (15.8%) and Zambia (14.3%) and lower for Kenya (8.7%). The prevalence of low birth weight was 17.2% (79/458) in DRC, 5.9% (36/609) in Kenya, and 9.1% (30/330) in Zambia. The prevalence of the maternal birth outcome of hypertensive disorders was very low: 0.2% (1/469) DRC, 0.8% (5/642) in Kenya, and 3.9% (13/335) in Zambia.

Crude association of malaria in the first trimester on maternal and birth outcomes

In the DRC, the crude prevalence of preterm birth (PD = 0.06 [99% CI: -0.03, 0.16]) and of low birth weight (PD = 0.07 [99% CI: -0.02, 0.16]) was elevated among women with malaria in the first trimester compared to women without malaria in the first trimester In Kenya, we saw higher crude prevalence of preterm birth (PD = 0.03 [99% CI: -0.03, 0.10]) and of anemia in late pregnancy (PD = 0.10 [99% CI: -0.01, 0.21]) among women with malaria in the first trimester. In Zambia, we found significant lower crude prevalence of perinatal mortality (PD = -0.06 [99% CI: -0.09, -0.02]). However, none of the 21 Zambian women with malaria in the first trimester had the birth outcome of perinatal mortality (**Table** 5.2).

We did not pool results from perinatal mortality because at least one of the countries had no cases of the outcome when stratified by malaria exposure. The results of the heterogeneity assessment supported pooling across countries for the crude prevalence ratio and crude prevalence difference for preterm birth, small for gestational age, and anemia in late pregnancy, and for the crude prevalence ratio for low birth weight (**Table** 5.3).

Malaria in the first trimester was associated with a higher prevalence of preterm birth among women from DRC, Kenya, and Zambia (summary PD = 0.03 [99% CI: -0.02, 0.08]). We observed a consistent elevated summary prevalence ratio and 99% confidence interval (summary PR = 1.38 [99% CI: 0.92, 2.07]). Malaria in the first trimester was not associated with changes in the prevalence of small for gestational age among women from all three sites (summary PD = -0.02 [99% CI: -0.07, 0.03]; summary PR = 0.87 [99% CI: 0.57, 1.33]). Malaria in the first trimester was associated with higher prevalence of low birth weight among women when pooled together (summary PR = 1.47 [99% CI: 0.91, 2.38]); the heterogeneity assessment did not support pooling for the low birth weight prevalence difference. Finally, malaria in the first trimester was associated with higher prevalence of anemia in late pregnancy (summary PD = 0.08 [99% CI: 0.00, 1.16]). We observed an elevated summary prevalence ratio for anemia (summary PR = 1.21 [99% CI: 0.95, 1.56]) (**Table** 5.3).

Adjusted effect of malaria in the first trimester on maternal and birth outcomes

After adjustment for age, education, socioeconomic status, malnutrition (with enrollment BMI at enrollment as a proxy), and season coincident with the first trimester as suggested by the DAG (**Figure** 5.3), we found higher prevalence of preterm delivery among Congolese women (aPD = 0.06 [99% CI: -0.04, 0.16]) but not among Kenyan (aPD = 0.03 [-0.04, 0.09]) or Zambian women (aPD = -0.01 [99% CI: -0.10, 0.20]) who had malaria in the first trimester compared to women who did not. We also saw an elevated prevalence of low birth weight among Congolese women (aPD = 0.07 [99% CI: -0.03, 0.16]) but not among Kenyan (aPD = 0.01 [-0.04, 0.06] or Zambian (aPD = -0.04 [99% CI: -0.13, 0.16]) who had malaria in the first trimester.

The prevalence of anemia in late pregnancy also was slightly higher among Kenyan (aPD = 0.05 [99% CI: -0.06, 0.17]), Zambian (aPD = 0.07 [99% CI: -0.12, 0.36]), and Congolese women (aPD = 0.04 [99% CI: -0.09, 0.16]) with malaria in the first trimester. In Zambia, we found a significantly lower prevalence of perinatal mortality among women with malaria in the first trimester (aPD = -0.06 [99% CI: -0.09, -0.03]), however all Zambian women who had malaria in the first trimester (n=21) did not have perinatal mortality. We did not find an effect of malaria in the first trimester on perinatal mortality among Congolese (aPD = 0.02 [99% CI: -0.04, 0.08]) or Kenyan women (aPD = 0.03 [99% CI: -0.02, 0.08]) (**Table** 5.4).

Discussion

We are the first study to estimate the effect of malaria in the first trimester on maternal and birth outcomes among a large number of women from multiple African countries and transmission settings. We found higher prevalences of preterm delivery and low birth weight among Congolese women with malaria in the first trimester, and higher prevalence of anemia in late pregnancy among Kenyan, Zambian, and Congolese women who had malaria in the first trimester. We additionally found a significantly lower prevalence of perinatal mortality among Zambian women with malaria in the first trimester, but all Zambian women with malaria in the first trimester did not have perinatal mortality, and we did not find this association among Congolese or Kenyan women.

Among the three study sites, we found higher prevalence of preterm delivery among Congolese and Kenyan women, but not among Zambian women with malaria in the first trimester. Research on the association of malaria in early pregnancy and

preterm delivery suggest conflicting relationships. Two studies assessing malaria before 24 weeks of gestation found an increased risk of preterm delivery[7] or an increased odds of preterm delivery among multigravidae only.[4] The last study assessed malaria in the first trimester among 273 Beninese women (of which 7.4% were primigravidae) and found no association with preterm birth.[92] We also used malaria in the first trimester as our exposure window but we focused primarily on primigravidae and had larger numbers of pregnant women followed in all three sites. We may have been able to detect a weak association between malaria in the first trimester and preterm delivery because DRC and Kenya had high malaria transmission (malaria in the first trimester prevalence 63.3% in DRC and 38.0% in Kenya), we focused on women who were the most vulnerable to malaria infection, and included large numbers of women in our analysis (469 in DRC and 642 in Kenya).

We additionally found higher prevalence of low birth weight among Congolese women with malaria in the first trimester, but not among Kenyan or Zambian women with malaria in the first trimester. While multiple studies have assessed the association of malaria in early pregnancy and low birth weight, the relationship is still unclear, with several finding higher risk and others finding no association.[11,85,92] Moeller et al. found an association of malaria infection before 15 weeks of gestation and lower birth weight among 138 Tanzanian women,[11] McGready et al., found no association of malaria in the first trimester infection (of either *P. falciparum* or *vivax*) and birth weight among 17,613 women who lived along the Thai Burmese border[85] and Accrombessi et al. also found no association of malaria in the first trimester infection and birth weight among 273 Beninese women.[92] Our focus on women who are most affected by malaria in

pregnancy (i.e., primigravidae) and the high malaria transmission burden may have allowed us to detect an association between malaria in the first trimester and higher prevalence of low birth weight among Congolese women.

Across all three countries with different transmission intensities, we saw elevated crude and adjusted prevalences of anemia in late pregnancy among women with malaria in the first trimester. We also found a significantly higher prevalence in anemia in late pregnancy among women with malaria in the first trimester in the pooled crude prevalence difference (with a consistent elevated pooled prevalence ratio). Most previous research assessing the association of malaria in early pregnancy and maternal anemia in late pregnancy or at delivery have found higher risk of the outcome, with only one study finding no association.[4,20,86,88,92] Our results confirm these findings although we did not see significantly higher crude or adjusted prevalences of anemia in late pregnancy when stratified by country. However, as our study population was restricted to women without severe maternal anemia (<7 g/dL) at enrollment, our findings were biased towards a null relationship and are non-generalizable to all nulliparous women.

We saw significant crude and adjusted lower prevalences of perinatal mortality among Zambia women with malaria in the first trimester. Only one study (in Burkina Faso) has assessed the association of malaria in the first trimester and perinatal mortality, and as no women with malaria in the first trimester had perinatal mortality and they were using incidence rate ratios, they did not calculate an effect measure.[86] Similarly, we also had few women in Zambia who had malaria in the first trimester (31 out of 1034 women from Burkina Faso and 21 out of 351 women from Zambia in our study), and among these women with malaria in the first trimester from either country,

none had perinatal mortality.[86] However, as we calculated both prevalence ratios and prevalence differences, we were able to calculate the prevalence difference despite zero outcomes of perinatal mortality among Zambian women with malaria in the first trimester. While we did find a significant association between malaria in the first trimester and lower prevalence of perinatal mortality among Zambia women, we did not find this association among Congolese or Kenyan women. Due to low numbers of Zambian women with malaria in the first trimester and possible selection bias caused by restricting the study population to women who delivered at 20 weeks of gestational age or greater, this result among Zambian women has strong limitations to reliability.

Similar to previous research on small for gestational age and malaria in early pregnancy (i.e., gestational age between 4-12 weeks, before 15 weeks, and before 24 weeks), we did not find an association between malaria in the first trimester and prevalence of small for gestational age.[7,10,92] No studies have assessed the association of malaria in early pregnancy and hypertensive disorders, and while we found a significantly lower crude prevalence of hypertensive disorders among Zambian women with malaria in the first trimester, all Zambia women with malaria in the first trimester did not have hypertensive disorders. The prevalence of hypertensive disorders was very low for all three countries ranging from 0.2% in DRC to 3.4% in Zambia, and thus we did not estimate the adjusted effect of malaria in the first trimester on hypertensive disorders.

A strength of this study was that we predominantly studied women with the highest risk of malaria in pregnancy and consequent negative health outcomes, i.e., women in their first pregnancy (primigravidae).[13,44] Over successive pregnancies,

women naturally acquire resistance to malaria in pregnancy by developing antibodies that inhibit binding of *P. falciparum*-infected erythrocytes to the placenta after a previous infection during pregnancy.[12,13] Thus, primigravidae lack this parity-dependent immunity that reduces placental and peripheral parasitemia.[13,44] While we studied nulliparous women, most (94%) of the women in our study were primigravidae.

Our study used DAGs as a framework to describe the causal relationship between the exposure of malaria in the first trimester and the adverse maternal or birth outcome. Based on these DAGs, the minimally sufficient adjustment set to estimate the total effect of malaria in the first trimester on any of the assessed maternal and birth outcomes was age, education, SES, season that coincided with the first trimester of pregnancy, malnutrition (used BMI as a proxy), and ITN use. We were limited to data collected by the ASPIRIN trial that focused on low-dose acetylsalicylic acid, and thus did not have data on ITN. However, as many pregnant women acquire ITNs through their first antenatal care visit, and women rarely use ITNs before and during their first pregnancy, the impact of ITN use on malaria in the first trimester among nulliparous women is likely to be low.[8,21,23]

Another strength was our use of the parametric g-computation to estimate the average causal effect of malaria in the first trimester on the assessed maternal and birth outcome. Using log binomial models to calculate adjusted prevalence differences frequently face issues with convergence. We instead used logistic regression as our model as logistic regression always converges. We were able to convert the predicted log-odds of outcome given everyone had malaria in the first trimester (or everyone was not exposed) to the predicted prevalence of outcome given everyone had malaria in the

first trimester (or everyone was not exposed). From these predicted prevalences, we took the mean to estimate the mean prevalence of the outcome given everyone was exposed (or not), and then calculated adjusted prevalence ratios and adjusted prevalence differences.

We thus estimated the adjusted effect of malaria in the first trimester on the assessed outcome. However, our goal was to estimate the causal effect of malaria in the first trimester on adverse maternal and birth outcomes. In order to interpret our results of our g-computation approach as an estimate of the average causal effect, we would need to assume counterfactual consistency, exchangeability, and positivity.[142] While we used the DAGs to identify confounders and adjusted for all confounders included in the ASPIRIN dataset, we did not adjust for ITNs or other unmeasured confounders.

Thus, we cannot assume that we had conditional mean exchangeability. Consequently, we also cannot assume that there is a non-zero probability of all values of the exposure conditional on the variables required for conditional mean exchangeability as we cannot justify our identifying and measuring all variables required for conditional mean exchangeability. Therefore, while our goal was to estimate the average causal effect of malaria in the first trimester on maternal and birth outcomes, we can only interpret our results as the adjusted effect of malaria in the first trimester on the assessed outcome.

In the first, large multi-site study of malaria in the first trimester, we used an efficient study design nested within a large clinical trial to estimate the effect of malaria in the first trimester on adverse maternal and birth outcomes across three transmission

settings among a large number of nulliparous women. We found higher prevalence of preterm delivery and low birth weight among Congolese women, and higher prevalence in the prevalence of anemia in late pregnancy among Congolese, Kenyan, and Zambian women.

In conclusion, we found that in these first-trimester infections are very common in high transmission settings and can lead to non-significant higher prevalence in preterm delivery, low birth weight, and anemia in late pregnancy. The higher prevalences of adverse maternal and birth outcomes associated with malaria in the first trimester suggest that strategies including screening in the first trimester are needed to reduce the impact of malaria in the first trimester.

Table 5.1: Characteristics of the study participant analysis population, stratified by country

Variable	DRC	KENYA	ZAMBIA
Randomized, N	469	642	335
Maternal age (years), N (%)			
< 20	396 (84.4)	298 (46.4)	196 (58.5)
20-29	66 (14.1)	340 (53.0)	136 (40.6)
> 29	7 (1.5)	4 (0.6)	3 (0.9)
Median (P25, P75)	18.0 (17.0, 18.0)	20.0 (18.0, 22.0)	19.0 (18.0. 21.0)
Projected gestation age at enrollment (weeks, days), N (%) [a]			
6, 0 - 7, 6	44 (9.4)	110 (17.1)	37 (11.0)
8, 0 - 9, 6	127 (27.1)	198 (30.8)	74 (22.1)
10, 0 - 10, 6	68 (14.5)	86 (13.4)	33 (9.9)
11, 0 - 11,6	81 (17.3)	85 (13.2)	49 (14.6)
12, 0 - 13, 6	149 (31.8)	163 (25.4)	142 (42.4)
Median (P25, P75)	10.9 (9.1, 12.3)	10.1 (8.3,12.0)	11.4 (9.1, 12.7)
Maternal education, N (%)			
No formal	76 (16.2)	1 (0.2)	11 (3.3)
Primary	226 (48.2)	42 (6.5)	31 (9.3)
Secondary	166 (35.4)	520 (81.0)	289 (86.3)
University +	1 (0.2)	79 (12.3)	4 (1.2)
Maternal height (cm)			
Mean (SD)	155.8 (6.6)	156.2 (8.8)	157.5 (6.4)
Median (P25, P75)	156.0 (151.0, 160.0)	157.1 (150.0, 162.4)	157.0 (154.0, 161.0)
Maternal weight (kg)			
Mean (SD)	50.5 (6.7)	56.4 (7.4)	54.6 (8.8)
Median (P25, P75)	50.0 (46.0, 55.0)	56.0 (51.0, 61.0)	54.0 (49.0, 59.0)
Maternal BMI (kg/m^2)			
Mean (SD)	20.8 (2.2)	23.3 (3.5)	22.0 (3.3)
Median (P25, P75)	20.5 (19.1, 22.1)	22.8 (20.8, 25.0)	21.6 (20.0, 23.5)
Antenatal care visits			
Mean (SD)	3.7 (1.1)	4.4 (1.3)	4.0 (0.8)
Median (P25, P75)	4.0 (3.0, 4.0)	4.0 (4,0, 5.0)	4.0 (4.0, 4.0)
Delivery attendant, N (%)			
Physician	11 (2.3)	25 (3.9)	24 (7.2)
Nurse/nurse midwife	409 (87.2)	550 (85.7)	295 (88.1)

Traditional birth attendant	42 (9.0)	47 (7.3)	5 (1.5)
Family/Self/Other	7 (1.5)	20 (3.1)	11 (3.3)
Delivery location, N (%)			
Hospital	62 (13.2)	141 (22.0)	136 (40.6)
Clinic/health center	350 (74.6)	406 (63.2)	184 (54.9)
Home/Other	57 (12.2)	95 (14.8)	15 (4.5)
Delivery mode, N (%)			
Vaginal	460 (98.1)	618 (96.3)	317 (94.6)
C-section	9 (1.9)	24 (3.7)	18 (5.4)

[a] Projected gestational age at enrollment developed from algorithm described in Hoffman et al., 2020

Table 5.2: Crude prevalence, prevalence ratios (PRs), crude prevalence differences (PDs), and 99% confidence intervals (CIs) for maternal and birth outcomes for nulliparous women by malaria in the first trimester status from the Democratic Republic of Congo, Kenya, and Zambia

	DEMOCRATIC REPUBLIC OF CONGO			KENYA			ZAMBIA		
	Prevalence (%, n/N)	PR (99% CI)	PD (99% CI)	Prevalence (%, n/N)	PR (99% CI)	PD (99% CI)	Prevalence (%, n/N)	PR (99% CI)	PD (99% CI)
Preterm birth									
Malaria +	21.9 (65/297)	1.39 (0.82, 2.38)	0.06 (-0.03, 0.16)	11.1 (27/244)	1.42 (0.75, 2.71)	0.03 (-0.03, 0.10)	4.8 (1/21)	0.68 (0.05, 8.87)	-0.02 (-0.15, 0.10)
Malaria -	15.7 (27/172)	REF	REF	7.8 (31/398)	REF	REF	7.0 (22/314)	REF	REF
Small for gestational age									
Malaria +	14.8 (42/283)	0.84 (0.47, 1.50)	-0.03 (-0.12, 0.07)	7.9 (18/227)	0.86 (0.42, 1.77)	-0.01 (-0.07, 0.05)	15.0 (3/20)	1.05 (0.25, 4.36)	0.01 (-0.20, 0.22)
Malaria -	17.6 (28/159)	REF	REF	9.2 (34/370)	REF	REF	14.2 (42/295)	REF	REF
Low birth weight									
Malaria +	19.9 (58/292)	1.57 (0.86, 2.88)	0.07 (-0.02, 0.16)	7.4 (17/231)	1.46 (0.64, 3.37)	0.02 (-0.03, 0.08)	4.8 (1/21)	0.51 (0.04, 6.53)	-0.05 (-0.17, 0.08)
Malaria -	12.7 (21/166)	REF	REF	5.0 (19/378)	REF	REF	9.4 (29/309)	REF	REF
Perinatal mortality									
Malaria +	7.4 (22/297)	1.27 (0.49, 3.30)	0.02 (-0.04, 0.08)	7.4 (18/244)	1.84 (0.78, 4.34)	0.03 (-0.02, 0.08)	0.0 (0/21)	NA	-0.06 (-0.09, -0.02)
Malaria -	5.8 (10/172)	REF	REF	4.0 (16/398)	REF	REF	9.4 (18/314)	REF	REF
Anemia in late pregnancy									
Malaria +	58.7 (168/286)	1.10 (0.88, 1.38)	0.05 (-0.07, 0.18)	41.5 (83/200)	1.31 (0.97, 1.77)	0.10 (-0.01, 0.21)	23.5 (4/17)	2.13 (0.63, 7.15)	0.12 (-0.15, 0.39)
Malaria -	53.3 (88/165)	REF	REF	31.8 (102/321)	REF	REF	11.1 (29/262)	REF	REF
Hypertensive disorders during pregnancy									
Malaria +	0.0 (0/297)	NA	-0.01 (-0.02, 0.01)	0.8 (2/244)	1.09 (0.10, 11.31)	0.00 (-0.02, 0.02)	0.0 (0/21)	NA	-0.04 (-0.07, -0.01)
Malaria -	0.6 (1/172)	REF	REF	0.8 (3/398)	REF	REF	4.1 (13/314)	REF	REF

Table 5.3: Crude pooled prevalence ratios (pooled PRs), crude pooled prevalence differences (pooled PDs), 99% confidence intervals (CIs), and corresponding I^2 values and 99% CI for maternal and birth outcomes for nulliparous women by malaria in the first trimester status from the Democratic Republic of Congo, Kenya, and Zambia

	I^2 (99% CI)	Pooled PR (99% CI)	I^2 (99% CI)	Pooled PD (99% CI)
Preterm birth				
Malaria + vs. Malaria -	0.0% (0.0, 80.4)	1.38 (0.92, 2.07)	0.0% (0.0, 94.7)	0.03 (-0.02, 0.08)
Small for gestational age				
Malaria + vs. Malaria -	0.0% (0.0, 27.8)	0.87 (0.57, 1.33)	0.0% (0.0, 49.8)	-0.02 (-0.07, 0.03)
Low birth weight				
Malaria + vs. Malaria -	0.0% (0.0, 91.7)	1.47 (0.91, 2.38)	48.9% (0.0, 89.9)	NA
Anemia in late pregnancy				
Malaria + vs. Malaria -	31.4% (0.0, 96.5)	1.21 (0.95, 1.56)	0.0% (0.0, 83.5)	0.08 (0.00, 1.16)

Table 5.4: Adjusted prevalence ratios (aPRs), adjusted prevalence differences (aPDs), and 99% confidence intervals (CIs) for maternal and birth outcomes for nulliparous women by malaria in the first trimester status in the Democratic Republic of Congo, Kenya, and Zambia

	DRC		KENYA		ZAMBIA	
	aPR (99% CI)	aPD (99% CI)	aPR (99% CI)	aPD (99% CI)	aPR (99% CI)	aPD (99% CI)
Preterm birth						
Malaria + vs. Malaria -	1.45 (0.80, 2.98)	0.06 (-0.04, 0.16)	1.31 (0.64, 2.65)	0.03 (-0.04, 0.09)	0.86 (0.00, 4.82)	-0.01 (-0.10, 0.20)
Small for gestational age						
Malaria + vs. Malaria -	0.76 (0.41, 1.46)	-0.05 (-0.16, 0.05)	0.82 (0.36, 1.62)	-0.02 (-0.08, 0.04)	1.12 (0.00, 3.44)	0.02 (-0.17, 0.29)
Low birth weight						
Malaria + vs. Malaria -	1.61 (0.86, 3.63)	0.07 (-0.03, 0.16)	1.15 (0.48, 2.79)	0.01 (-0.04, 0.06)	0.56 (0.00, 3.07)	-0.04 (-0.13, 0.16)
Perinatal mortality						
Malaria + vs. Malaria -	1.46 (0.52, 5.81)	0.02 (-0.04, 0.08)	1.67 (0.64, 4.61)	0.03 (-0.02, 0.08)	NA	-0.06 (-0.09, -0.03)
Anemia in late pregnancy						
Malaria + vs. Malaria -	1.07 (0.86, 1.35)	0.04 (-0.09, 0.16)	1.16 (0.83, 1.60)	0.05 (-0.06, 0.17)	1.62 (0.00, 5.03)	0.07 (-0.12, 0.36)

Note: All models were adjusted for age, education, socioeconomic status, season coincident with the first trimester, and body-mass index.

99% CIs were developed using bootstrapping with 10,000 resamples and selecting the 50th and 9950th value.

Figure 5.1: Map of malaria transmission intensity based on study site
The study locations are located in Nord-Ubangi and Sud-Ubangi provinces in the Democratic Republic of the Congo, in Bungoma, Busia, and Kakamega provinces in Kenya, and in Kafue and Chongwe districts in Lusaka province in Zambia. Malaria transmission is modeled from the *Plasmodium falciparum* parasite rate among children aged 2-10 (PfPR 2-10) in 2015 and ranges from 0.6 in Nord-Ubangi and Sud-Ubangi provinces in the Democratic Republic of the Congo to 0.0 in Lusaka province in Zambia. Data on the modeled PfPR 2-10 was obtained from the Malaria Atlas Project.[143]

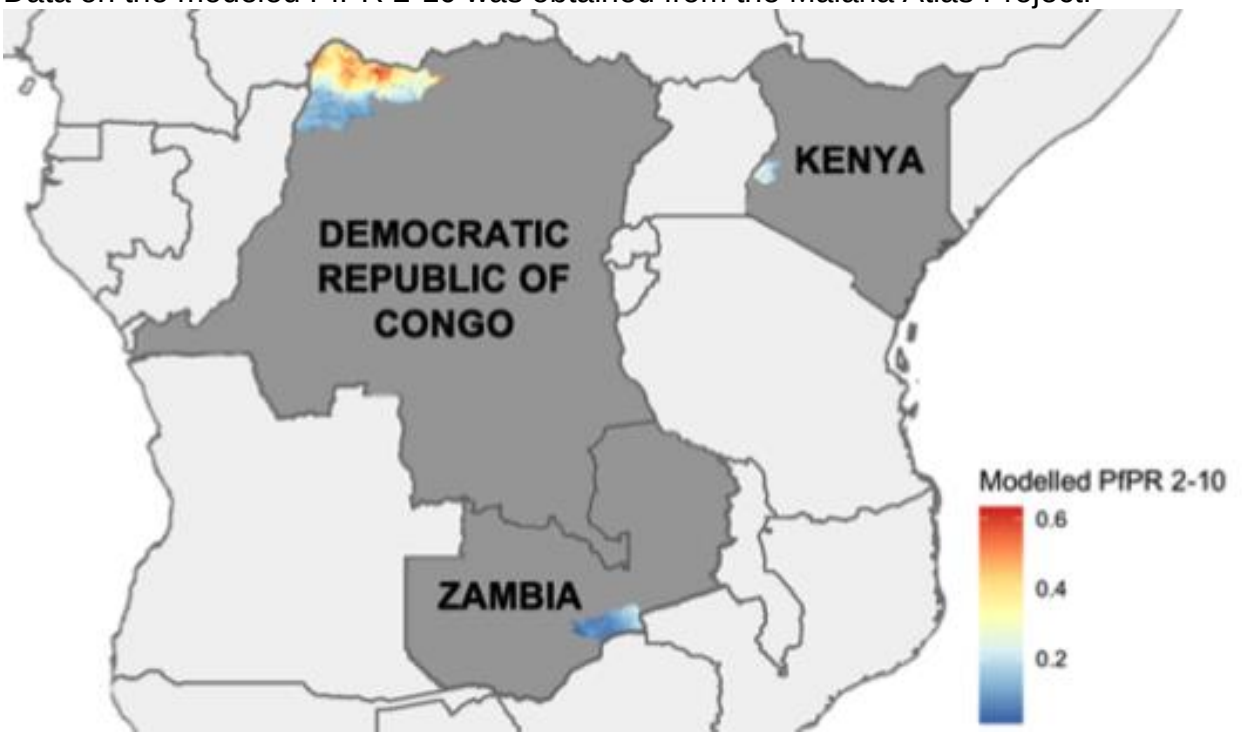


Figure 5.2: Study population of malaria analysis sub-study
The ASPIRIN trial included 3800 women from Democratic Republic of the Congo (DRC), Kenya, and Zambia. From these women, we took a convenience sample of 1446 women for the analysis population of the malaria sub-study: 469 from DRC, 642 from Kenya, and 335 from Zambia.

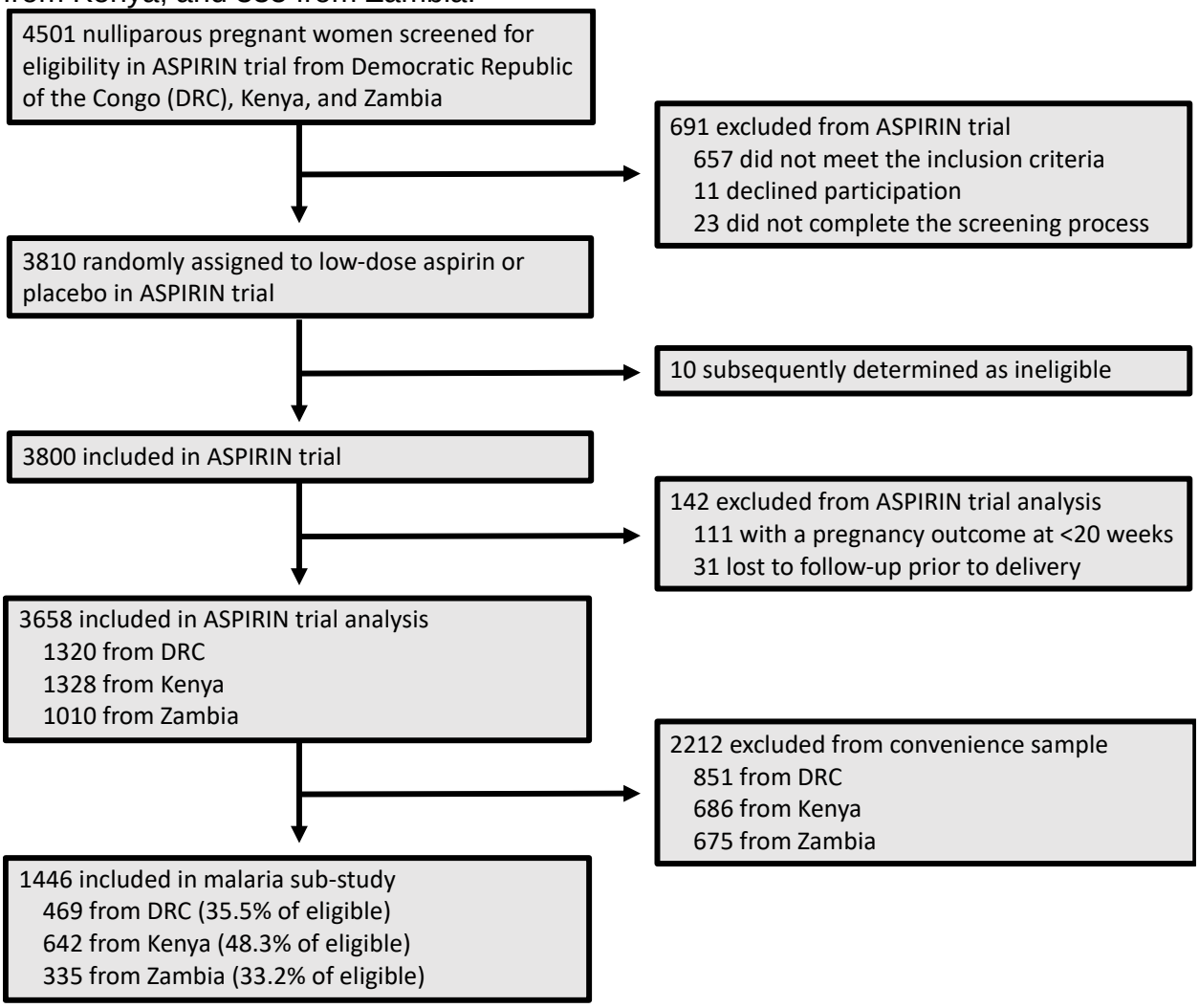
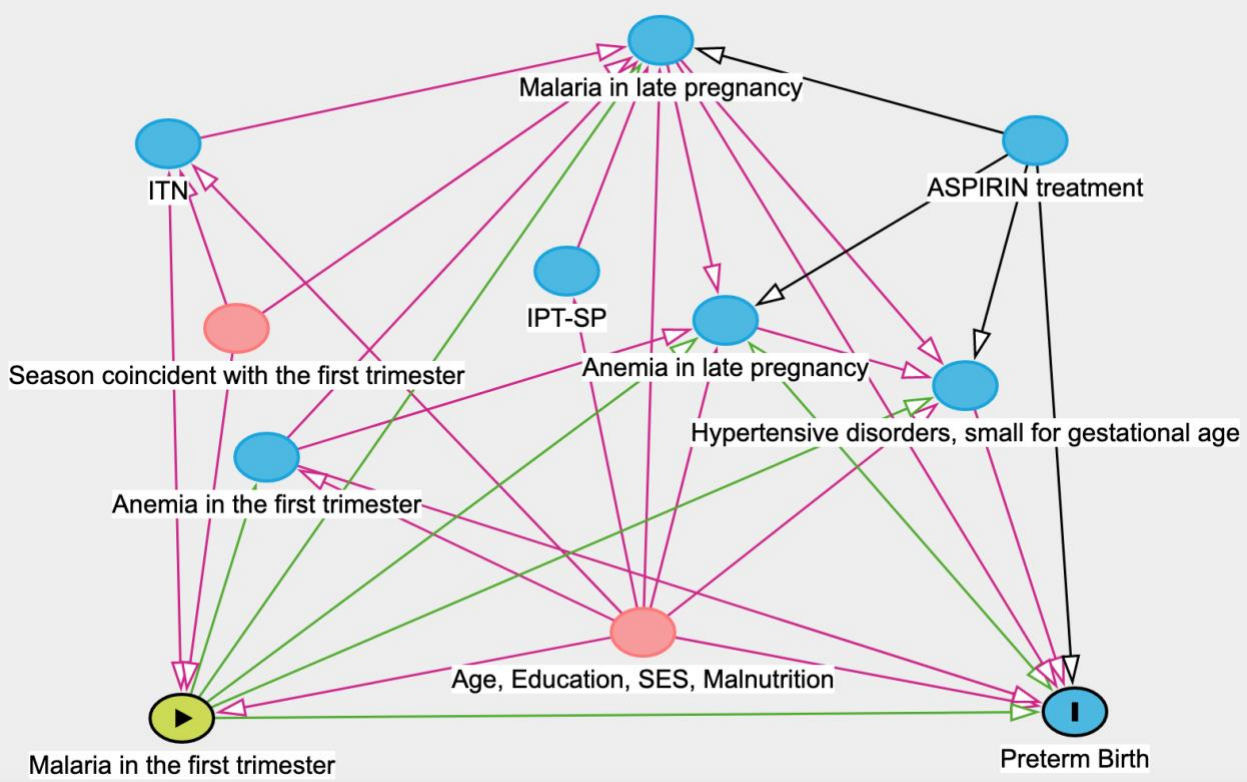


Figure 5.3: DAG of the relationship between the exposure of malaria in the first trimester and the outcome of preterm birth

The minimally sufficient adjustment set to determine the total effect of malaria in the first trimester on preterm birth is season coincident with the first trimester, age, education, socioeconomic status (SES), malnutrition (used a proxy of maternal BMI), and insecticide-treated nets (ITN) use.



CHAPTER VI: CONCLUSIONS

Summary of Findings

This dissertation was motivated by the current gap in the literature studying malaria in the first trimester. Although the first trimester might represent a critical time for intervention to prevent the negative consequences of malaria in pregnancy, few studies have studied malaria in the first trimester. We characterized what factors are associated with increased likelihood of malaria in the first trimester and estimated causal effects of malaria in the first trimester on adverse maternal and birth outcomes. We hypothesized that lower overall educational attainment (no secondary education) would be associated with increased likelihood of malaria in the first trimester, and that malaria in the first trimester would increase the prevalence of all the adverse maternal and birth outcomes assessed. Malaria in the first trimester was studied among a large number of women from sites within three countries with different malaria transmission levels among nulliparous women.

This study was nested within the NICHD Global Network's trial of low-dose aspirin for the prevention of preterm delivery in nulliparous women with a singleton pregnancy (the ASPIRIN Trial). The ASPIRIN trial recruited nulliparous women in their first trimester between March 2016 and April 2019 at seven research sites in six countries, including three in sub-Saharan Africa: Democratic Republic of the Congo,

Kenya, and Zambia. Dried blood spots were collected from participants at enrollment and were retrospectively tested for *Plasmodium falciparum* parasites. After linking quantitative polymerase chain reaction results on malaria in the first trimester to extant ASPIRIN trial data, we used crude and pooled effect measures to determine predictors of malaria in the first trimester and used the parametric g-computation and directed acyclic graphs to estimate its effects on maternal and birth outcomes.

For the predictors of malaria in the first trimester in Aim 1, we found the prevalence of malaria in the first trimester varied across sites: 62.9% in the DRC, 37.8% in Kenya, and 6.3% in Zambia. Consistent with our hypothesis, we found that lower overall educational attainment was associated with higher prevalence of malaria in the first trimester in pooled estimates developed from women in all three countries. We also found that younger age and shorter height were associated with higher prevalence of malaria in the first trimester among Kenyan women.

For the effects on maternal and birth outcomes of malaria in the first trimester in Aim 2, we found higher prevalences of preterm delivery and low birth weight among Congolese women with malaria in the first trimester, and higher prevalence of anemia in late pregnancy among Kenyan, Zambian, and Congolese women who had malaria in the first trimester. We additionally found a significantly lower prevalence of perinatal mortality among Zambian women with malaria in the first trimester, but all Zambian women with malaria in the first trimester did not have perinatal mortality, and we did not find this association among Congolese or Kenyan women.

The dissertation findings suggest that the burden of malaria in the first trimester varies greatly by site and differences in findings on predictors varied by country.

However, when pooled across all three sites, lower overall educational attainment was associated with higher prevalence of malaria in the first trimester. In addition, in areas of high transmission, women who had malaria in the first trimester had higher prevalences of adverse birth outcomes, and in all three sites regardless of transmission intensity, malaria in the first trimester was associated with higher prevalence of anemia in late pregnancy. This research provides a rationale to conduct more research identifying actionable predictors and to improve prevention and treatment of malaria among women in their first trimester

Strengths and Limitations

Strengths and limitations of each aim are detailed below across five areas in epidemiology: confounding, measurement, missingness, selection, and generalizability.

Confounding

For the first aim of predictors, we calculated crude and pooled crude associations of the assessed predictor and malaria in the first trimester. As we were determining only the marginal associations, we did not need to control for confounding variables.

For the second aim of maternal and birth outcomes, we used DAGs to identify the minimally sufficient adjustment set of covariates. Our goal was to estimate the causal effect of malaria in the first trimester on adverse maternal and birth outcomes. In order to interpret our results of our g-computation approach as an estimate of the average causal effect, we would need to assume mean conditional exchangeability (i.e., the potential outcomes were independent of the observed exposure given the covariates). While we used DAGs to identify confounders and adjusted for all confounders included in the ASPIRIN dataset, we did not adjust for ITNs or other

unmeasured confounders. Thus, we cannot assume that we had conditional mean exchangeability.

Measurement

In both aims, we used a qPCR assay to detect malaria in the first trimester, but as pregnant women frequently have very low parasitemia levels, it is possible we did not detect some infections. However, PCR is very sensitive and can detect infections missed by common diagnostic tools such as RDTs and microscopy. However, the clinical significance of these subpatent or submicroscopic infections is still a matter of some debate.[36,45] While the outcome was malaria infection in the first trimester, as qPCR is able to detect very low levels of parasite nucleic acids, we could have detected residuals from a non-viable sequestered parasite.[36]

In Aim 1, we assessed the predictors of maternal age, maternal height, maternal BMI, maternal education, socioeconomic status, and season coincident with the first trimester. The first five of the above listed predictors were recorded by the ASPIRIN trial at enrollment. This data which was reported by each of the sites to the Global Network Data Coordinating Center (RTI International) who validated a random 5% of the actual data collected by chart review to ensure data quality.

We may have misclassified maternal BMI at enrollment as it is calculated using maternal weight which increases over pregnancy. While maternal weight was recorded between 6 weeks 0 days and 13 weeks 6 days of gestational age, pregnant women who were enrolled later in gestational age could have had increased maternal weight due to pregnancy. However, any measurement bias is likely minimal as women were enrolled

in the first trimester and thus before women in resource-limited countries typically gain significant weight due to pregnancy.[144]

The birth outcome of hypertensive disorders included diagnosis of preeclampsia at the time of birth admission which was determined by measuring blood pressure and evaluating proteinuria. However, at the clinics and health centers included in this study, assessing blood pressure and proteinuria was frequently not routine procedure for birth admission, leading to women with preeclampsia being misclassified as women without preeclampsia. However, to address this misclassification, the definition of hypertension was intentionally wide to try to capture some of these misclassified women.

Missingness

Across the two aims, a convenience sample of DBS were processed and included in the analysis. Therefore, between one-third to one-half of participants who were eligible for this sub-study were included in our data analysis. In addition, we further restricted the study population from Aim 1 to only include women who provided any post-baseline outcome data and who delivered at 20 weeks of gestational age or greater for Aim 2. While we did not assess the malaria in the first trimester status of those not included in our convenience sample or the effects of malaria in the first trimester among those who delivered before 20 weeks of gestational age, our study population numbers were reasonably large compared to previous studies.

In addition, for both aims, we used complete case analysis and thus limited comparisons and models to observations without missing data for each variable and covariate. For Aim 1, there were no missing data except for socioeconomic status (missing n=53), and similarly for Aim 2, there were no missing covariate data except for

socioeconomic status (missing n=40). However, for Aim 2, while there were no missing data for preterm birth, perinatal mortality, or hypertension, 6% of women were missing data for small for gestational age, 3% for low birth weight, and 13.5% for anemia in late pregnancy. Thus, it is possible that these birth outcomes were not missing completely at random, and thus we may have produced biased estimates, especially for anemia in late pregnancy.

Selection

Selection criteria for the ASPIRIN trial included women who had no more than two previous first-trimester pregnancy losses and excluded women with multiple gestations, severe anemia at screening, and hypertensive disorders at screening. By excluding women with more than two previous first-trimester pregnancy losses, we likely excluded women with a higher likelihood of miscarrying before 20 weeks of gestation. Thus, we likely reduced the number of women included at enrollment who were excluded from the analysis population because they did not deliver at 20 weeks of gestation or greater. Also, women with multiple gestations are more likely to have birth complications, and thus we likely reduced the overall prevalence of adverse birth outcomes by only including singleton pregnancies. By restricting to women without severe anemia at screening, and considering that malaria infection can lead to anemia, we likely did not enroll many women with malaria infection because they had severe anemia. In addition, as one of our maternal outcomes was anemia in late pregnancy, by excluding women with severe maternal anemia at enrollment who likely had malaria in the first trimester, our results were biased to find a null relationship. Finally, by excluding

women with hypertensive disorders at screening, we likely reduced the number of women with hypertensive disorders during pregnancy in our analysis population.

Generalizability

This dissertation study was based in three countries with varying malaria transmission levels and included nulliparous pregnant women with singleton pregnancies. Thus, our country-specific findings may be relevant to nulliparous pregnant women with singleton pregnancies from other areas of sub-Saharan Africa with similar transmission intensities. While we studied nulliparous women, over 90% of our study population were primigravidae. Although we studied women with the highest risk of malaria in pregnancy and consequent negative health outcomes, our results are not generalizable to women with lower risk of malaria in pregnancy (i.e., multigravidae). Overall, our study demonstrates how leveraging existing clinical trials can lead to inexpensive measurement of malaria in the first trimester which can be used to characterize predictors and estimate effects on maternal and birth outcomes of malaria in the first trimester in other study settings.

Public Health Implications and Future Directions

This is the first study to assess malaria in the first trimester at multiple sites, and we were able to determine that the prevalence of malaria in the first trimester varied by transmission intensity; in the DRC where the transmission intensity is high, the prevalence of malaria in the first trimester was also high, and in Zambia where the transmission intensity was low, the prevalence of malaria in the first trimester was also low. Consistent with previous research, lower overall educational attainment (no secondary education) was associated with higher prevalence of malaria in the first

trimester. In addition, other predictors of malaria in the first trimester varied by country, and malaria in the first trimester can lead to higher prevalences in preterm birth, low birth weight, and anemia in late pregnancy.

As lower overall educational attainment (no secondary education) was associated with higher prevalence of malaria in the first trimester, public health officials should target ITN distribution strategies towards women with lower overall educational attainment. In addition, given the differences in our findings on most predictors by country, more research is needed to identify actionable predictors to focus interventions and target women at highest risk of malaria in the first trimester. In addition, the detrimental effects of malaria in the first trimester on preterm birth, low birth weight, and anemia in late pregnancy suggest that interventions should be expanded to focus on prevention and treatment of malaria among primigravidae women in the first trimester and even preconception. Examples include providing ITNs to all women before pregnancy instead of at the first ANC visit, shifting ANC to begin in the first trimester, and driving research developments on prophylactic malarial medications for use in the first trimester.

Future research includes increasing sample sizes to improve power to detect differences in maternal and birth outcomes. In addition, more studies need to assess predictors of malaria in the first trimester in order to identify actionable predictors. Future studies should also assess predictors and the effects on maternal and birth outcomes of malaria in the first trimester among women of all gravidae. Finally, malaria in the first trimester is very common in high transmission settings despite implementation of current WHO recommendations of diagnosing and treating malaria,

IPT-SP, and ITNs, suggesting a need for stronger interventions. Future studies can assess detection and treatment methods of malaria in pregnancy in order to reduce the prevalence of malaria in the first trimester

Using the first large-scale multi-site study of malaria in the first trimester, we were able to assess predictors and the effects on maternal and birth outcomes of malaria in the first trimester at three sites with different transmission settings. We found that malaria in the first trimester is very common in high transmission settings and can lead to higher prevalences of preterm delivery, low birth weight, and anemia in late pregnancy. The high prevalence of infections in high-transmission areas along with associations of malaria in the first trimester with higher prevalences of adverse maternal and birth outcomes suggest that screening women early in pregnancy may be needed to reduce the impact of malaria in the first trimester.

APPENDIX

Appendix Information for Chapter IV

Projected gestational age at enrollment was developed from algorithm as previously described in Hoffman et al., 2020.[19] In short, it was developed by taking the date of randomization, subtracting the value – projected expected delivery date (EDD) minus 280 days – and then dividing by 7. The definition of projected EDD varied depending on whether it was the same as the ultrasound EDD or the last menstrual period (LMP) EDD.

The projected EDD equals to ultrasound EDD under the following conditions: (1) If LMP was unknown or gestational age (GA) at randomization according to LMP was less than zero; (2) If the GA at randomization according to LMP was between 0 weeks and 0 dates to 8 weeks and 6 days, and the difference between GA at randomization according to LMP and GA at randomization according to ultrasound was greater than 5; (3) If the GA at randomization according to LMP was between 9 weeks and 9 days to 13 weeks and 6 days, and the difference between GA at randomization according to LMP and GA at randomization according to ultrasound was greater than 7; and (4) If the GA at randomization according to LMP was 14 weeks 0 days or greater.

The projected EDD equals the LMP EDD under the following conditions: (1) If the GA at randomization according to LMP was between 0 weeks and 0 dates to 8 weeks and 6 days, and the difference between GA at randomization according to LMP and GA at randomization according to ultrasound was 5 or less; and (2) If the GA at randomization according to LMP was between 9 weeks and 9 days to 13 weeks and 6

days, and the difference between GA at randomization according to LMP and GA at randomization according to ultrasound was 7 or less.

The Global Network Socioeconomic Status Index was used to assess socioeconomic status and is calculated by determining the sum score of the number of 10 specific items owned by the household that was converted to a country-specific SES score.[136] These country-specific SES score range from 0 to 100.[136] The mean and standard deviation (SD) of SES index scores were similar for the 50,000 households the GN SES Index was developed from and for the women included in this malaria sub-study. For Congolese women, the mean was 11.01 and SD was 9.33 for all households, and the mean was 12.96 and SD was 9.61 for household included in this malaria sub-study.[136] For Kenyan women, the mean was 17.13 and SD was 18.24 for all households, and the mean was 16.96 and SD was 17.37.[136] For Zambian women, the mean was 35.26 and the SD was 22.13 for all households, and the mean was 43.61 and SD was 26.08.[136] However, for all household, about one-third were nulliparous while in this sub-study all participants were nulliparous.

Appendix Information for Chapter V

There were 92 (6.4% of total sample) women missing data for small for gestational age (27 (5.8%) from DRC, 45 (7.0%) from Kenya, and 20 (6.0%) from Zambia), 49 (3.4% of total sample) missing data for low birth weight (11 (2.3%) from DRC, 33 (5.1%) from Kenya, and 5 (1.5%) from Zambia), and 195 (13.5%) missing data for anemia in late pregnancy (18 (3.8%) from DRC, 121 (18.8%) from Kenya, and 56 (16.7%) from Zambia). There were no missing covariate data except for 40 (2.8% of

total sample) women missing socioeconomic status data (15 (3.2%) from DRC, 25

(3.9%) from Kenya).

Figure A.1: DAG of the relationship between the exposure of malaria in the first trimester and the outcome of low birth weight

The minimally sufficient adjustment set to determine the total effect of malaria in the first trimester on low birth weight is season coincident with the first trimester, age, education, maternal socioeconomic status (SES), malnutrition (used a proxy of maternal BMI), and insecticide-treated nets (ITN) use.

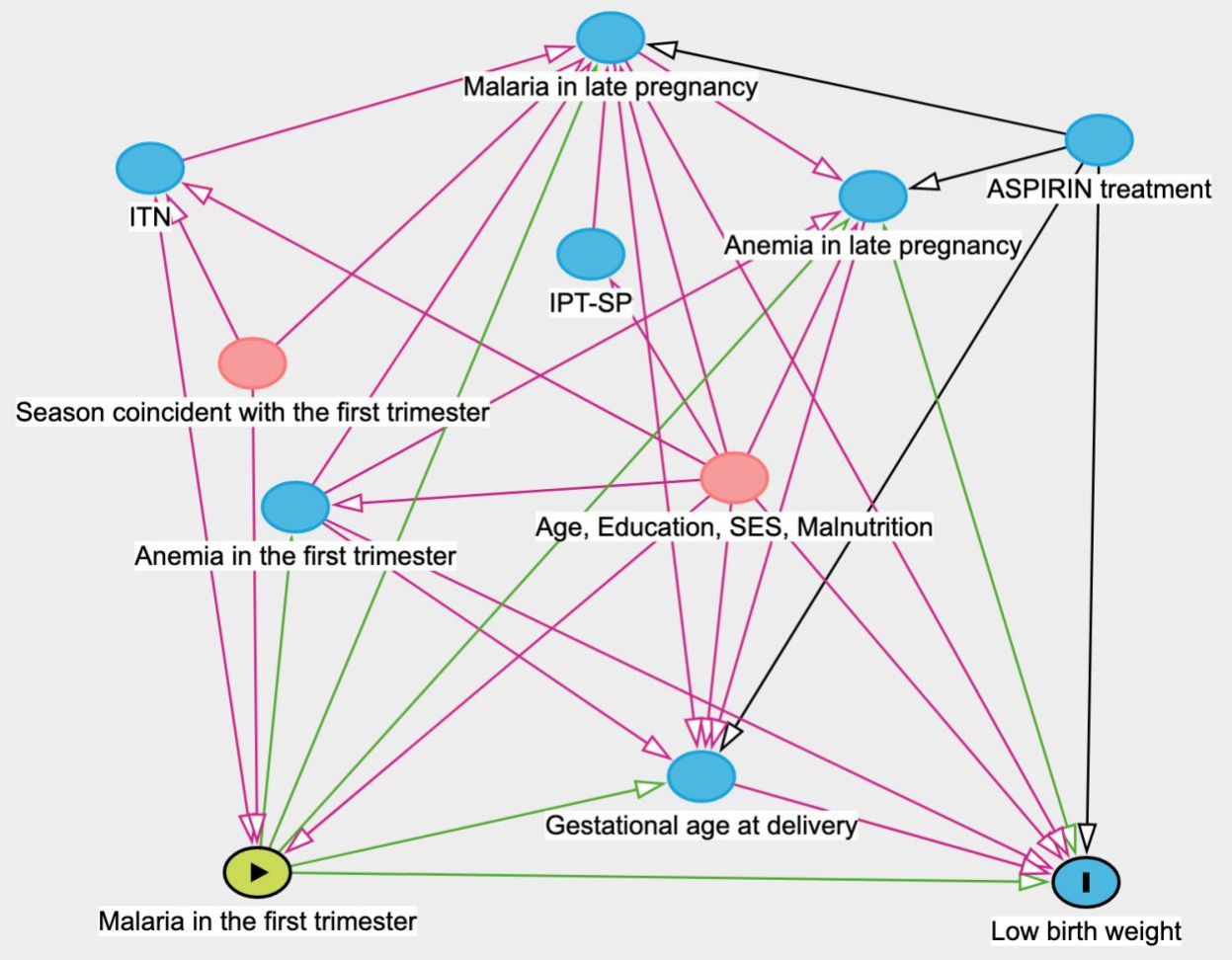


Figure A.2: DAG of the relationship between the exposure of malaria in the first trimester and the outcome of small for gestational age

The minimally sufficient adjustment set to determine the total effect of malaria in the first trimester on small for gestational age is season coincident with the first trimester, maternal age, maternal education, maternal socioeconomic status (SES), malnutrition (used a proxy of maternal BMI), and insecticide-treated nets (ITN) use.

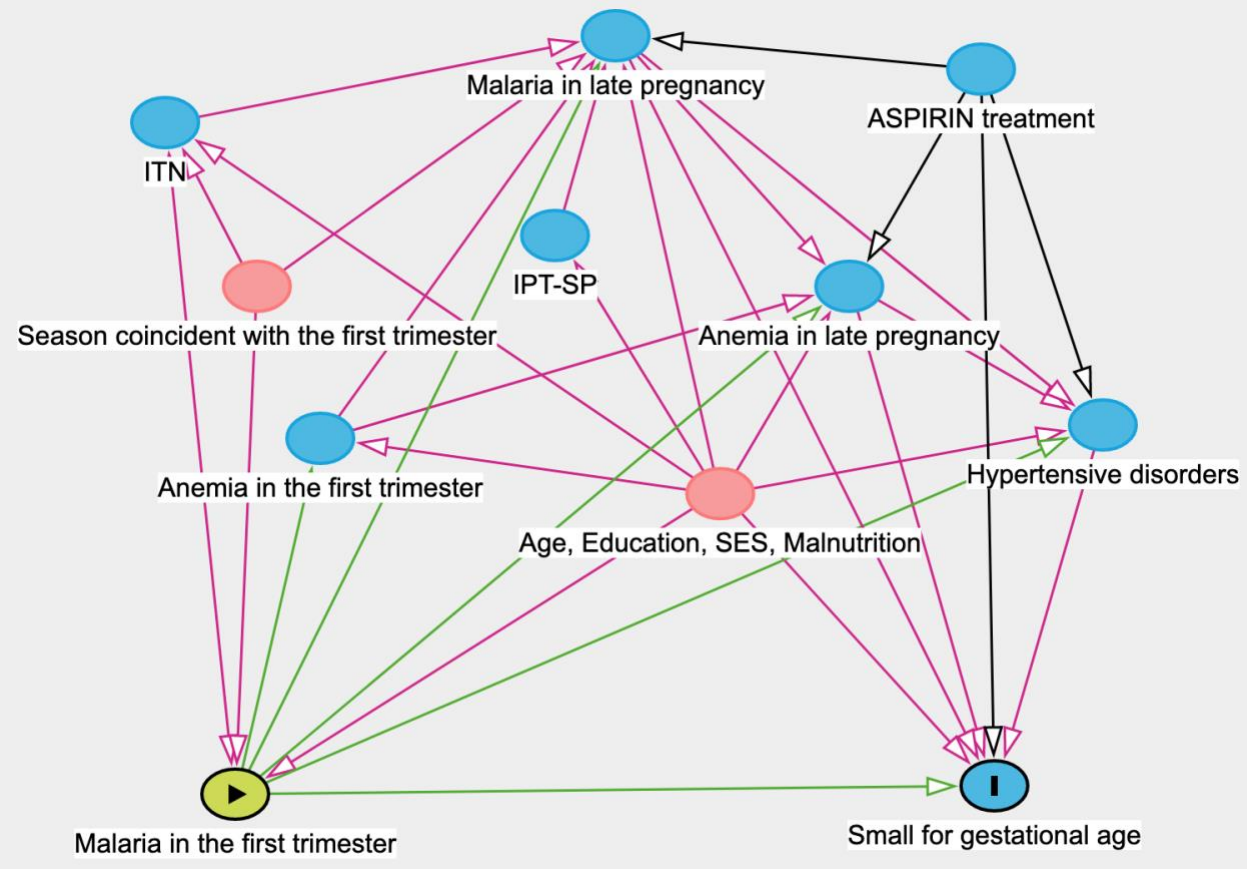


Figure A.3: DAG of the relationship between the exposure of malaria in the first trimester and the outcome of perinatal mortality

The minimally sufficient adjustment set to determine the total effect of malaria in the first trimester on perinatal mortality is season coincident with the first trimester, maternal age, maternal education, maternal socioeconomic status (SES), malnutrition (used a proxy of maternal BMI), and insecticide-treated nets (ITN) use.

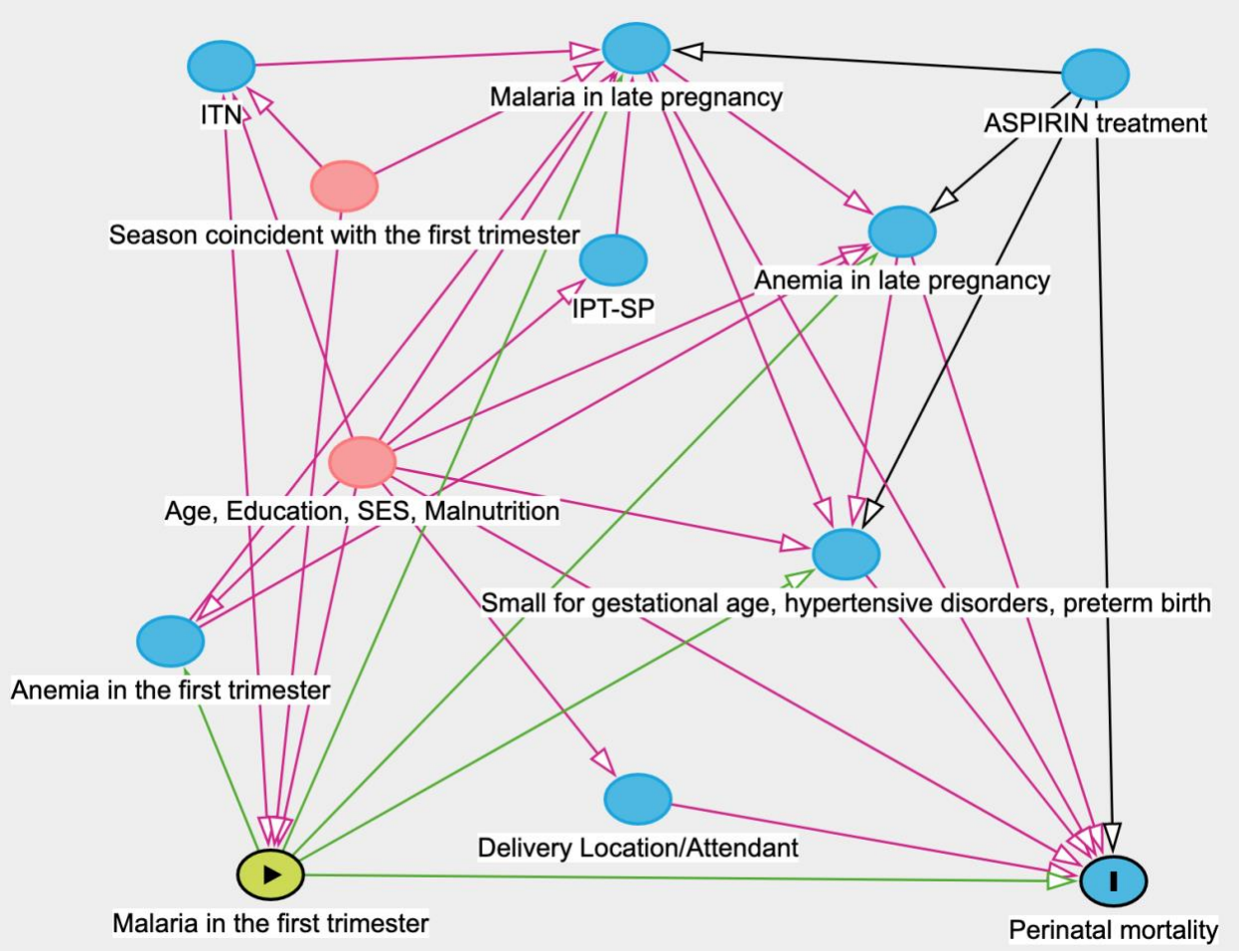
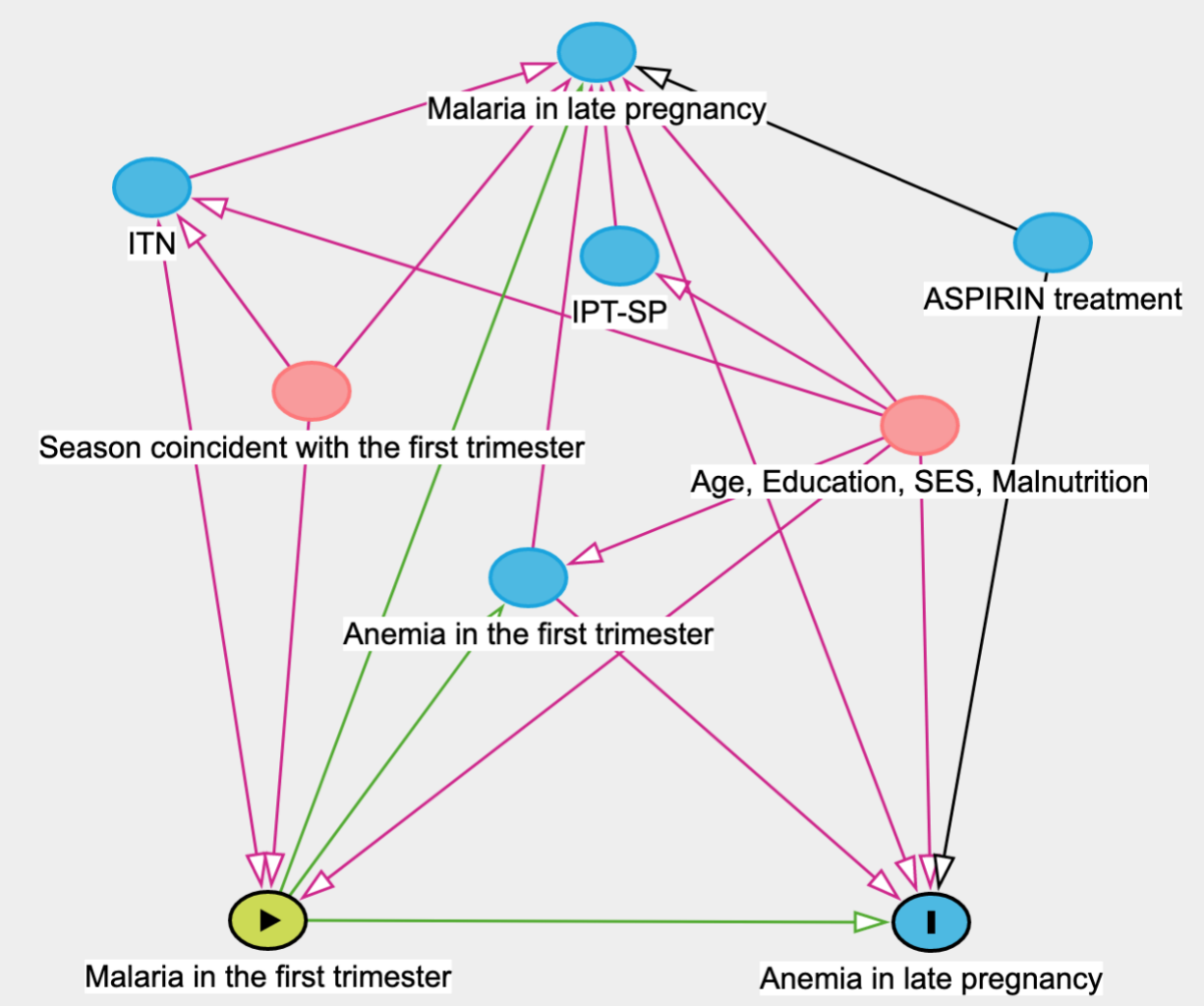


Figure A.4: DAG of the relationship between the exposure of malaria in the first trimester and the outcome of anemia in late pregnancy.
The minimally sufficient adjustment set to determine the total effect of malaria in the first trimester on anemia in late pregnancy is season coincident with the first trimester, age, education, socioeconomic status (SES), malnutrition (used a proxy of maternal BMI), and insecticide-treated nets (ITN) use.



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